THE COAGULATION OF BLOOD

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Methods of Study

edited by

LEANDRO M TOCANTINS, M D

Prepared with the help and under the sponsorship of the Panel on Blood Coagulation of the Committee on Medicine and Surgery of the National Academy of Sciences National Research Council



GRUNE & STRATTON 1955

NEW YORK LONDON

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Library of Congress Catalog Card No 54 12319

Printed and bound in U.S.A

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PREFACE

AT ALMOST EVERY MEETING of the Panel on Blood Coagulation of the National Academy of Sciences National Research Council the members are called upon to decide on the merits of proposals for research in this field The impression has often been left in the minds of the members that many such proposals have come from persons who, though actuated by a sincere desire to investigate some phase of the subject of clotting were often unacquainted with the methods involved It seemed to the members of the panel that a few such workers might be helped if a compendium of these methods were prepared to which investigators could refer for general guidance From the first it was pointed out that this should be no attempt to set down such methods rigidly as standard " Even if this were possible it would, of course not be desirable Since the principal functions of the Panel are to judge, encourage and facilitate research the concept of setting definitive standards becomes naturally abhorrent

It was with these thoughts in mind that the Panel on Blood Coagulation commissioned me to prepare such a compendium A general outline of the topics was drawn up and submitted to the Panel members and other interested workers each of whom assumed the responsibility of writing the topics assigned to him The project was promptly endorsed by the Chairman of the Division of Medical Sciences of the National Academy of Sciences National Research Council Dr Milton C Winternitz and subsequently by his successor Dr R Keith Cannan The members of the Panel who participated in the deliberations and in the preparation of the compendium were

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From the first Dr Seegers the Chairman of the Panel supported the effort enthusiastically and contributed much helpful advice and guidance

The decision to include any method rested principally on the familiarity of the contributors with the technic and their estimate of its importance It is fully realized that other technics exist unquestionably as important and useful as those described but limitations of space have made it impossible to include all but a few of those originally selected Dr

FOREWORD

THE PHENOMENON OF BLOOD CLOTTING has attracted physiologists and biochemists in the past with a superficial aspect of simplicity From the nature of the effect, it appeared to the observer to be an example of a biologic process that, by reason of its accessibility to controlled study, should be capable of precise physical characterization and quantitative definition

*Intensive study over many years has advanced our knowledge im*measurably At the same time it has destroyed the illusion of simplicity What appeared at first sight to be a straightforward enzymatic modification of a protein has emerged as a highly integrated system of interacting accelerators and inhibitors With this growth in complexity of mechanism there has inevitably developed a confusion of tongues and of technics that has been the despair of those not intimately working in the field

In view of the mounting clinical interest in the clotting process the National Research Council appointed a Panel on Blood Coagulation in 1951 It was the responsibility of this Panel to interpret to the Committee on Surgery the contemporary status of the problem and to advise it on ways of fostering research leading to a better understanding of the mechanism of coagulation In particular the Panel was invited to attempt to clarify and simplify current nomenclature and to examine the practical problem of devising methods of predicting thrombotic tendencies in man

The Panel has organized a series of conferences in which these objectives have been advanced It has also devoted particular attention to the elucidation and refinement of technical procedures and this book is one product of these efforts It is not offered as an authoritative selection of *standard methods—the subject is too fluid for standardization—but rather* as a compilation of what appeared to the Panel to be the more satisfactory procedures for general use described in sufficient technical detail to be of value to workers in the field

This volume was written primarily by the members of the National Research Council's Panel on Blood Coagulation including the liaison members from the Canadian Defence Research Board with the help of their associates and other interested investigators The Division of Medical Sciences of the National Research Council wishes to express its sincere appreciation to all who participated in this work for their excellent contributions and especially to Dr Leandro M Tocantins who as editor has devoted so much of his time to this enterprise and has shown such excellent judgment in the organization of this book

R KEITH CANNAN CHAIRMAN
Division of Medical Sciences

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R. KEITH CANNAN CHAIRMAN
Division of Medical Sciences

INTRODUCTION

WORK RELATED TO BLOOD COAGULATION and allied problems now covers a broad field of activity encompassing clinical applications vascular research pharmacology, studies on the formed elements of the blood metabolism the fractionation of plasma the industrial production of plasma fractions the chemistry of the blood clotting mechanisms and other endeavors By looking at this from a broad perspective one sees that greatest attention has centered on the chemistry of the blood coagulation mechanisms This is understandable in terms of the great difficulties presented by such studies and the strategic nature of the useful information derived from such work

The advances of the past decade now furnish a rather clear picture of the fundamental nature of the blood coagulation mechanisms As a matter of convenience one may classify the interactions into four main events This classification is based on the simple fundamental fact that prothrombin gives rise to thrombin if inhibitors do not prevent it This is followed by the transformation of fibrinogen to fibrin and the subsequent neutralization of thrombin activity by antithrombin

In the activation of prothrombin a large number of substances are important Among these we may list calcium thromboplastin Ac globulin the platelets with all of their components the antihemophilic factor the plasma thromboplastin component and other activators All of these are in one way or another related to the activation of prothrombin Many of these themselves undergo transformations before prothrombin is activated Others change only after some thrombin has formed

The inhibition of prothrombin activation is very closely linked with the prothrombin activators but it is convenient to consider it separately in terms of a number of specific inhibitors such as heparin and its co-factor antithromboplastin and other inhibitors The general plan follows patterns in which inhibitors interact with those substances classified as activators of prothrombin

The action of thrombin on fibrinogen may be considered the main event in the blood coagulation mechanisms when judged by what one sees and when judged by what seems to be the ultimate purpose of all the events It is plain however that thrombin is involved in its own formation on the basis of autocatalysis and interaction with other substances of the

Margaret Sloan, Executive Assistant to the Chairman of the Division of Medical Sciences displayed a continued interest in furthering the work The Publishers with their already established reputation of encouraging medical research, have been of much assistance to the Committee in fulfilling our objectives

Philadelphia, Pennsylvania LEANDRO M TOCANTINS
December, 1954

time in collecting the material and completing the editorial work His own technical skills are greatly admired by his colleagues and his long experience in active and productive research and editorial work is reflected in this effort His willingness to contribute so generously made it easy to have the cooperation of others who also gave much time and effort in their support of the project

WALTER H SEEGERS *Chairman*
Panel on Blood Coagulation

blood coagulation mechanisms It is an enzyme and as such is capable of acting on synthetic substrates, and can even dissolve the fibrin clot in due time under appropriate circumstances

The neutralization of thrombin activity involves the interaction of thrombin with more than one substance of the blood and by several mechanisms For example, there is adsorption of thrombin on fibrin A plasma protein interacts chemically with neutralized thrombin activity Heparin and a plasma co factor together may interfere with the interaction of thrombin and fibrinogen, and during the activation of prothrombin there are interactions which greatly speed up the neutralization of thrombin activity

Here it is not necessary to discuss the fibrinolytic system for the above is only intended to give a glimpse of the many variables involved in the study of the blood coagulation mechanisms These must be taken into account in future metabolic studies and in any application of our fundamental knowledge In an effort to create a higher order of insight than previously known attempts have been made to introduce quantitative methods These are the cornerstone and basis of all concepts we may consider reliable Many times these quantitative methods turn out to be only approximations of the original ideal Just the same they serve their useful purpose in what amounts to successive approximations to true and reliable quantitative measurements Some technics have probably arrived at that state of perfection while others are on their way, and we can assume that more are going to be developed Methods have also been developed for the purification of certain substances concerned with the blood coagulation mechanisms If these technics are the cornerstones of our knowledge then it is of utmost importance that they be understood and used with knowledge of their meaning and limitations

Members of the Panel on Blood Coagulation in the Division of Medical Sciences of the National Research Council considered that the information now available on quantitative technics or any other technics related to the chemistry of blood coagulation is widely scattered in the literature and not easily accessible It seemed obvious that it would be helpful to collect a number of useful descriptions under one cover It was however evident that this work could not be comprehensive in scope that it would have to be limited and that one could not easily escape leaving some readers perplexed as to what might have been the guiding principle in the selection of the material It is an offering which stands on its own on the basis that several active investigators regard this as most helpful in their work and assume and hope that others will likewise find the information useful

The Panel on Blood Coagulation was fortunate to find a member of its own group, Leandro Tocantins willing and able to spend his valuable

CHAPTER I

PROCESSING OF BLOOD, PREPARATION OF GLASSWARE AND REAGENTS

1 The Collection of Blood for Studies on Coagulation

L M TOCANTINS

It is not generally realized by most students of blood coagulation and those charged with the responsibility of clinical measurements of the rate of blood clotting that the mode of collection of the blood sample affects the results of this work considerably It may be worthwhile therefore to state in detail what we consider to be minimum standards for the conditions surrounding the collection and storage of blood from the vessels of man and experimental animals These remarks apply solely to the collection of blood for studies on blood coagulation and its various factors and not necessarily to the collection of blood for biochemical or cytological studies The fact that a defective collection technique will often be satisfactory for the latter studies has often misled many research and clinical workers into the belief that the same applies to studies on blood coagulation

1 Site for Collection of Blood A readily accessible vein of good caliber such as that of the forearm in men or of the leg or arm vein in dogs is adequate The hair over the area should be clipped if it interferes with visualization and approach to the vein but the skin itself should not be injured during the clipping or shaving The skin overlying the vein should be cleaned with water and alcohol In order to be sure of a clean rapid venepuncture the vein should be distended by applying a tourniquet proximal to the site of the puncture The tourniquet should be adjusted critically so that the arterial flow is adequate but the venous return is cut off Between one and two minutes should elapse before the vein is punctured to insure an adequate supply of blood and a taut distended readily punctured vein The blood should be aspirated into the syringe or allowed to flow out of the vein into a vessel at the rate of no less than 0 5 ml per second The rate of aspiration of the blood should be adjusted to the rate of flow so

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that the negative pressure applied by the piston is not in great excess of that needed to aspirate the blood, for, in such case air bubbles will enter the syringe between the piston and barrel or between the tip of the syringe and the hub of the needle

2 Needles The needle should be sharp with a bevel no longer than 3 mm and the gauge should be preferably 18 or at the minimum, 20 When ever possible it is preferable to work with greater gauges (17 or 16) No needle smaller than 20 should be used

3 Syringes Tight fitting syringes coated on the internal surface with silicone or mineral oil should be used If an anticoagulant is to be added to the blood the correct amount of the chemical in as small a volume as pos sible should be placed in the syringe, thus allowing the blood to come into contact with the anticoagulant immediately after it leaves the vessel It is desirable that at least two syringes be employed the first for the collection of 1 or 2 ml of blood to be discarded and replaced by a second syringe containing the anticoagulant After the required amount of blood has been aspirated the syringe should be removed and tilted four or five times to insure mixture of the anticoagulant and blood

4 Silicone coated glassware should be used for the centrifugation and storing of blood and plasma The syringe is emptied of blood by placing the tip of the syringe against the side of the siliconized tube and allowing the blood to run down (and not be squirted) on the side of the tube without frothing or excessive turbulence The maximum g and time employed for centrifugation should be stated The temperature in the centrifuge should not rise above 30 C during centrifugation and it is best kept at between 5 and 10°C

If plasma is to be separated this should be done immediately after collection of the blood If platelet poor plasma is desired only the upper three fourths of the plasma layer after centrifugation should be aspirated Siliconized glassware should be used in pipetting the plasma from one cen trifuge tube to another, and no reflux of plasma from the pipette back to the tube should be allowed

A specimen of blood unsuitable for clotting studies may result if any or all of the following occur

1 If there is difficulty in entering the vein and more than one trial is made at puncturing it If such is the case the fact should be noted by the operator in reporting the results

2 If the supply of blood in the veins is so restricted that a rapid aspiration is impossible This may be due to malposition or obstruction of the needle or inadequate filling of the vein with blood because of defective application of the tourniquet

3 *Entrance* of air bubbles into the syringe while the blood is being

withdrawn One or two air bubbles are permissible since these may come from the tip of the syringe or the needle itself Repeated entrance of bubbles into the syringe during aspiration is objectionable, since it interferes with the blood silicone interface by enlarging the air blood interface

4 Slow aspiration of blood into the syringe lengthens the time that the blood is out of the vessels without being adequately mixed with the anticoagulant The use of large volume syringes (30 ml or more) is undesirable since it lengthens the time required for aspirating enough blood to fill the syringe

2 Selection of Surfaces for Needles, Syringes, Collection Sets, Containers and Test Tubes To Be Used in Clotting Studies

L B JAQUES

Many surfaces have been and are used for this purpose Selection is usually made (consciously or unconsciously) on the basis of availability of material availability of suitable form cost machinability transparency ease of cleaning restoration of surface after u e effect on clotting These properties are summarized in table 1 + indicates favourable − indicates unfavourable property of particular surface ± property is favourable or unfavourable depending on use Cleaning is discussed separately With all surfaces a very great problem (sometimes insurmountable) is removing the cleaning agent used

Details for application[8] of a series of coatings which can be sterilized by autoclaving and are of sufficient variety to cover all laboratory needs are given below

Application of Silicone (for glassware) The vapor treating material (Dri Film 9977) lends itself readily to the siliconization of large amounts of equipment in a minimum of time All glassware must be properly washed with soap and water 24 hours before application of the silicone After washing the glassware is thoroughly rinsed in warm tap water followed by hot pyrogen free distilled water It is then placed in a hot air oven and dried for 2 hours at 120 C Following this it is allowed to stand overnight at room temperature in order to equilibrate with the moisture in the atmosphere This overnight equilibration is absolutely essential as the vapor will not react with or adhere to a completely dry surface The glassware is put

TABLE 1 *Surfaces for Coatings*

Surface	Availability	Forms	Cost	Machinability, Application etc	Pliability	Transparency	Ease of Cleaning	Restoration of Surface	Effect on Clotting	Remarks and References
Metals Steel aluminum copper brass tin pewter nickel & alloys silver gold platinum	+ Generally available	+ Needles in steel & noble metals (rare) containers in limited forms	− depends on metal and amount of machining required	+ Chief advantage. Can be accurately machined	± rigid	− Opaque used in taking blood no visible control in containers observation must be from above	− can be cleaned where access for scouring and polishing. Difficult to use on lumen of small orifices needles etc as non visible inspection	? Possible where scouring polishing. Doubtful when this is not possible	+ Noble metals believed inert. Probably true of most metals provided shavings not left from machining and surface polished which is not possible in many uses	Hewson[1] obtained spectacular results with pewter & porcelain containers blood obtained with bistoury
Glass Soft Hard Quartz	+ Generally available	+ containers & tubing in great variety	+ Cheap. Quartz expensive	+ can be worked by any lab worker	± rigid fragile	+ transparent ease of observation & control	+ Easily cleaned	+ Readily restored but any variation in techniques may introduce change in surface	− Marked acceleration of clotting. Soft glass believed worse. Quartz inert	a
Paraffin liquid solid vaseline	+ generally available purity?		+ Cheap	+ Can be applied direct or solution (benzene) to most glass surfaces	±	+ transparent ease of observation & control	+ Surface is removed by organic solvents & the reapplied each time		+ Inert	
Cellulose Collodion Cellophane	+ Generally available	+ Collodion in solution − Cellophane tubes & sheets	+ Cheap	+ Collodion can be applied to containers pipette glass surfaces	− fragile	+ Transparent ease of observation & control	+	+	+ Inert	

End th l m (ll g test t be)	+ g erally labl	− Jug lar vei f d gs & rabbits	− Coat f imal	−	− frag le & permeable	− Semiopaqu	− N t reco ral le	+ 1 t	
Plastic r bbe	+ g rally labl	± t bing	+	± l mited	+ plast le	+ paq	+ d fficult to ure clea lin ss	+ most plastics ar i t t t imp ities may itiate use n t ly R b be is v ry un satisf ctory because f (1) subsits ces f m cu ng (2) d fficult to m re lea of blood after use *Other g re ers s f te fac which ca b applied t a y situation* [4]	Il les used s goose tract as t be Selected repre se tative plastics ha been list d Th use f ppl ca ti n and wid ra g d form make the plastics the most usef l type d s rface
polyth l t ld l te				l mited			f l		
Sil co	G Elec D Film 9977 9987	test-t bes M d to peci ficati s F lm to pply to ll glas r f ces i l po		C n be ma chi ed C n be us d to co r y glass s f ce	r gid −	Tra pare t Tra pare t	Easily cl ed wh s ocess to polishi g R m ed by 10% KOH & reapplied each tim		
Laq ihco	G n Elec *81202* Sil co e Resl Dow C ng P n Cl	P ma t *S f ce t sat*m t f oces t l tai l ss steel pa t Sol ti to pply to eedles		B ked t 230 C f 3 hrs	d rabl	−	Eas ly clea ed (l fe-o 150) / C n be removed with trong alkali and reapplied		
A q d 2C	A m r & C			S ppli r face for needles & th m tal parts w th ar w diam et	−	−	R mo ed with soap d water a d pplied ach tim		
T fl n	Dupo t C	S rf ce co t i g f r m tals		C be m hi ed b t h per as f ce applied to m tal bak d nd pol hed	r gid		Easily lea ed wh e access to polishi g / ?		

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Lampe t E D phys k lis h Se t des Bl tge i gsp obl m d ihre praktische Red t ng 127 pp Leip ig Geo g Thi m 1931
[3] Toca t ns L R l t o f cont t g urf ce to coag l ti n Am J Physi l *145* 67 1945
Li t J Th coll cted papers f J ph B ron List r v l I Oxford Cl re d n Press 1909
Roch w E G J q es L B a d Brow I S l co e in the Preserv t n of the F rmed Elem nts d f th Protei f the Blood N ti nal Red Cross 1949 p. 303
[4] Tullis J L d Roch w E G S l cones Blood *7* 850 1952

in cardboard cartons or other similar containers and placed under a chemical hood About 30 cc of Dri Film (No 9977) is placed in a gas washing bottle and air is bubbled slowly through it The resultant vapor is directed through a rubber tube and glass nozzle into each of the cartons containing glassware The tube should only be held in each carton long enough for the operator to count slowly to six The gas bottle is then disconnected and a stream of filtered compressed air is directed into each carton for the same period of time in order to displace the extra methyl chlorosilane vapor The glassware is then removed from the cartons rinsed three times with hot pyrogen free distilled water to free it of the hydrochloric acid which has been generated It is now ready for sterilization or immediate use as desired

Application of Arquad (for needles) Needles after cleaning are boiled in a 1 per cent solution of Arquad for two minutes, then rinsed and allowed to dry

Application of Lacquer Silicone (for accessible metal surfaces) The materials to be treated are prepared by dipping or brushing with the resin solution and are allowed to drain They should be placed in a vertical position to allow complete drainage They are left in this position for 30 minutes, allowing the resin to air dry so that it will not blister when it is permanently baked on The air dried pieces are then baked in an oven at 230°C for three hours, after which they are ready for use Following 150 or more exposures to blood, the previous coating may be removed by treatment with strong alkali These baked on surfaces are hard and durable Although they can be scratched, they are not ordinarily damaged by the usual laboratory manipulations

Application of Teflon (for accessible metal surfaces) This plastic may be applied as finely divided particles suspended in an appropriate dispersion agent and is poured or sprayed over the metal surface The resultant surface, after drying and heating, is polished and remains as a semi permanent coating

3 Preparation of Glassware

L. B JAQUES

All glassware should be first washed thoroughly with soap and water, rinsed repeatedly with tap water, and after rinsing with distilled water dried and then inspected Any showing cracks and scratches must be discarded Detergents should not be used for washing since these have a harmful effect on leucocytes

Further treatment depends on the measurement being made Many procedures require treatment with chromate-sulphuric acid cleaning mixture This removes fat by oxidation but will fix protein in situ Also it is very difficult to remove the traces of chromic acid Treatment with chromate cleaning solution is done in one of two ways Some prepare the cleaning solution by pouring 1 liter of concentrated sulphuric acid into 35 ml saturated sodium dichromate The glassware is allowed to sit in this overnight Others prepare a diluted solution of sodium dichromate-sulphuric acid Articles are boiled in this for 30 minutes

Alternative procedures for final preparation of glassware recommended by different workers are

1 treatment with chromate-sulphuric cleaning solution followed by rinsing 20 times with distilled water

2 treatment with chromate-sulphuric acid cleaning solution rinsing in tap-water boiling in distilled water, rinsing again with distilled water

3 treatment with chromate-nitric acid and extensive rinsing

4 treatment with 40 per cent NaOH rinsing treatment with chromate cleaning solution extensive rinsing

5 after washing with soap and water the glassware is rinsed thoroughly with distilled water then rinsed twice with alcohol twice with ether and dried

6 after washing with soapy water the glassware is rinsed thoroughly with tap water then distilled water and dried

4 Removal and Application of Silicone

L M TOCANTINS

Siliconized glassware (pipets, tubes, syringes) should be cleaned by first washing in warm water then immersing the glassware in the following mixture

Technical grade acetone	2 liters
H_2O	2 liters
NaOH (solid)	150 Gm

Dissolve the NaOH in about 300 ml of H_2O before adding to the acetone and H_2O Allow the glassware to remain in the solution overnight, then remove the glassware rinse thoroughly with H_2O and dry in the oven Alternatively the well rinsed glassware may be immersed in a solution of equal parts of technical grade acetone and 5 25 per cent sodium hypochlorite (Janox Cleaner R W Hoffman Co Philadelphia, Penna) with 25 grams NaOH added per liter of solution Eight hours are necessary to remove the silicone The glassware is then removed from this solution rinsed well (a brush may be necessary in some instances) and dried in the oven

Siliconizing

A solution of 1 part Dri Film 9987 (General Electric) and 4 parts of petroleum ether is used to coat the glass surfaces which must be clean and dry Technic

Test Tubes One tube is filled to the top with the silicone petroleum ether solution the contents poured into a second from the second to the third and so on until all are finished As the solution is poured from one tube to another it is rotated, so that the entire inner surface of the tube is coated with the silicone When the tubes are all coated they are filled with distilled water and allowed to stand from 15 to 30 minutes They are then drained and inverted and put in the oven to dry at 120°C from 60 to 90 minutes They are then tightly covered dated and stored in a clean dry closet until used

Pipets Draw the solution up into the pipet with a small rubber bulb dipping the pipet about two inches into the solution (so as to siliconize the outer surface) Draw the solution only about an inch below the mouth end Drain the pipets well and rinse thoroughly with distilled water and place in oven (120 C) to dry for about 60 minutes

Syringes A few cc of the solution are drawn into the syringe which is then inverted and the plunger pulled down slowly as the silicone coats the

barrel sides This is repeated two or three times so as to coat the plunger The silicone solution is then forced out, the barrel and plunger separated and thoroughly rinsed with distilled water and placed in the oven to dry Do not put syringe together until completely dry

Needles are siliconized in the same manner and at the same time Merely place the needle on the syringe tip at the beginning of the process before aspirating the silicone solution

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L M TOCANTINS

Siliconized glassware (pipets tubes, syringes) should be cleaned by first washing in warm water then immersing the glassware in the following mixture

Technical grade acetone	2 liters
H_2O	2 liters
NaOH (solid)	150 Gm

Dissolve the NaOH in about 300 ml of H_2O before adding to the acetone and H_2O Allow the glassware to remain in the solution overnight then remove the glassware rinse thoroughly with H_2O and dry in the oven Alternatively the well rinsed glassware may be immersed in a solution of equal parts of technical grade acetone and 5 25 per cent sodium hypochlorite (Janox Cleaner R W Hoffman Co Philadelphia Penna) with 25 grams NaOH added per liter of solution Eight hours are necessary to remove the silicone The glassware is then removed from this solution rinsed well (a brush may be necessary in some instances) and dried in the oven

SILICONIZING

A solution of 1 part Dri Film 9987 (General Electric) and 4 parts of petroleum ether is used to coat the glass surfaces, which must be clean and dry Technic

Test Tubes One tube is filled to the top with the silicone petroleum ether solution, the contents poured into a second from the second to the third and so on until all are finished As the solution is poured from one tube to another it is rotated so that the entire inner surface of the tube is coated with the silicone When the tubes are all coated they are filled with distilled water and allowed to stand from 15 to 30 minutes They are then drained and inverted and put in the oven to dry at 120 C from 60 to 90 minutes They are then tightly covered, dated and stored in a clean, dry closet until used

Pipets Draw the solution up into the pipet with a small rubber bulb dipping the pipet about two inches into the solution (so as to siliconize the outer surface) Draw the solution only about an inch below the mouth end Drain the pipets well and rinse thoroughly with distilled water and place in oven (120 C) to dry for about 60 minutes

Syringes A few cc of the solution are drawn into the syringe which is then inverted and the plunger pulled down slowly as the silicone coats the

6 Buffer Solutions of Imidazole (pH range 6 2–7 8) for Studies on Blood Coagulation (Mertz and Owen)

Adapted by R R HOLBURN

Prepare the following stock solutions

1 0 1 N HCl (adjust pH to 1 08 ± 0 1)

2 0 2 M Imidazole solution Dissolve 1 36 Gm of Imidazole powder (Edcan Co Norwalk Conn) in distilled H_2O to a total volume of 100 ml

In order to obtain solutions of the following pH the corresponding amounts stated below must be mixed and diluted with H_2O to a volume of 100 ml

pH	6.2	6 4	6 6	6 8	7 0	7 2	7 4	7 6	7.8
ml of Stock HCl Solution	42 9	39 8	35 5	30 4	24 3	18 6	13 6	9 3	6 0
ml of Stock Imidazole Solution	25	25	25	25	25	25	25	25	25

After mixture the pH of each solution should be checked with a pH meter

REFERENCE

Mertz E T and Owen C A Imidazole buffer Its use in blood clotting studies Proc Soc Exp Biol & Med 43 204 1940

5 List of Anticoagulants Employed in Studies on Blood Coagulation

R R HOLBURN

N	Compo d	Form l	P r C t Solu tion	Prepar tion Gm /100 ml H_2O	V l f S l t n to Vol of Blood
1	Sodium Citrate	$C_6H_5Na_3O_7$ $2H_2O$	3 2 3 8 19 0 38 0	3 2 Gm /100 ml H_2O 3 8 Gm /100 ml H_2O 19 Gm /100 ml H_2O 38 Gm /100 ml H_2O	1 to 9 1 to 9 1 to 49 1 to 99
2	Sodium Oxalate	$Na_2C_2O_4$	1 34	1 34 Gm /100 ml H_2O	1 to 9
3	Potassium Oxalate	$K_2C_2O_4$	1 4	1 4 Gm /100 ml H_2O	1 to 9
4	Lithium Oxalate	$Li_2C_2O_4$	1 4	1 4 Gm /100 ml H_2O	1 to 9
5	Oxalate Mixture Ammonium Oxalate Potassium Oxalate	 $(NH_4)_2C_2O_4$ $K_2C_2O_4$	2 0	1 2 Gm + 0 8 Gm /100 ml H_2O	1 to 9
6	Mixture Potassium Oxalate Sodium Fluoride	 $K_2C_2O_4$ NaF	20 0	10 Gm + 10 Gm /100 ml H_2O	1 to 19
7	Heparin	—	1 0	*1 Gm /100 ml H_2O*	*1 to 9*
8	Disodium sequestrene	$C_{10}H_{14}N_2Na_2O_8$	10 0	10 Gm /100 ml H O	1 to 9
9	Sodium Fluoride	NaF	40 0	40 Gm /100 ml H O	1 to 99
10	Potassium Fluoride	KF $2H_2O$	40 0	40 Gm /100 ml H_2O	1 to 99

CHAPTER II

INTRODUCTORY EXERCISES

L. M TOCANTINS

In this chapter are grouped what may be called the calisthenics for beginners in work on blood coagulation The exercises are designed to ac quaint the neophyte or near neophyte with the language and facts of the subject Since they have not been arranged in the order of simplicity, it may be wise if the inexperienced worker selects the simplest exercise and progresses gradually to the more complex ones It is the custom in our laboratory to have a technical worker spend from two to three months per forming these exercises and submitting the data to the senior workers in the department It is only after such a period of apprenticeship that we feel the technical worker may become sufficiently acquainted with the funda mental facts of blood coagulation to render his (or her) help effective and valuable At this stage of their training it is insisted that they do not read texts or articles on blood coagulation This is for the purpose of providing them with a stock of knowledge of these facts before they are plunged into the treacherous currents of the theory and terminology of blood coagulation

EXERCISE No 1 *Effect on Clotting Time of Changing the Volume of the Blood While Maintaining the Diameter of the Tube Constant*

Five uncoated glass and five silicone-coated tubes of 13 mm i d were selected To each group of 5 was added 0 5 1 0 1 5 2 0 and 3 0 ml of blood from a normal subject collected with the silicone technic The tubes were stoppered and kept at 38 C

Results

Tube N	1	2	3	4	5
Amount of Blood (ml)	0 5	1 0	1 5	2 0	3 0
Clotting Time (mins)					
Glass	10	15	16 5	21	21 5
Silicone	20	35	36	40	42

The rate of blood coagulation in glass or silicone coated surfaces in creases as the volume of the blood is increased

7 Estimation of Per cent Concentration of Plasma in a Clotting Mixture

L M TOCANTINS

The actual concentration of plasma separated from a sample of citrated (or oxalated) blood and the final plasma concentration in a clotting mixture may be determined as follows Measure the hematocrit in the citrated blood Estimate the concentration of the removed plasma making allowance for the volume of the anticoagulant added To determine the plasma concentration in the clotting mixture divide the volume of plasma by the total volume of the mixture and multiply by the citrated plasma concentration

Example

Hematocrit = 37 on blood collected with one part 38 per cent sodium citrate to 99 parts of blood Sixth three per cent of the blood sample is therefore plasma In the 63 parts of the plasma there is one part of citrate solution Therefore in order to calculate the actual plasma concentration in the original sample

$$\frac{62}{63} \times 100 = 98\ 4\%\ \text{plasma concentration}$$

In order to calculate the final concentration of plasma in a clotting mixture of 0 35 ml plasma 0 165 ml 0 85 per cent NaCl 0 035 ml 0 2 M $CaCl_2$

$$\frac{\text{Plasma vol}}{\text{Total Vol}} \times 98\ 4 = \frac{35}{55} \times 98\ 4$$

$$= 62\ 6\%\ \text{plasma concentration in clotting mixture}$$

Results

Tube No.	1	2	3	4	5
pH	6.4	6.8	7.2	7.5	7.9
Clotting Time (secs)	29	26	19	22	30

Raising or lowering of pH beyond 7.2 slows the rate of clotting of activated plasma

EXERCISE No 5 *Effect of Concentration of NaCl on Clotting Time of non activated Plasma*

Clotting Mixture 0.1 ml NaCl solution (variable per cent concentration)
0.1 ml Human Citrated Plasma
0.1 ml 0.02 M $CaCl_2$

Glass tubes 38 C

Results

Tube No	NaCl Solution (% Concentration)	Clotting Time (secs)
1	0.01	520
2	0.025	515
3	0.05	510
4	0.10	500
5	0.25	475
6	0.5	460
7	0.85	430
8	0.95	490
9	1.0	620
10	1.5	925
11	2.0	1 180
12	8.5	>21 600

NaCl solution of 0.85 per cent concentration seems to supply the optimal conditions for coagulation

EXERCISE No 6 *Effect of Different Surfaces on Rate of Coagulation and Clot Retraction of Normal Blood*

Blood was collected with the silicone technic and 1 ml was placed in each tube with a surface as indicated below

Type of Surface	Glass	Paraffin	Collod	Silicon
Clotting Time (mins)	6½	15	17	28
Serum expressed in 2 hours (% of clot volume)	32	40	0	50

EXERCISE No 2 *Effect on the Rate of Blood Coagulation of Changing the Diameter of the Tube While Maintaining the Volume of the Blood Constant*

Four uncoated glass and four silicone coated tubes of the following internal diameter 7 mm , 10 mm , 13 mm , 15 mm , were selected To each was added 1 ml of blood from a normal subject, collected with the silicone technic The tubes were stoppered and kept at 38 C

Results

Tub No	1	2	3	4
Internal Diameter (mm)	7	10	13	15
Clotting Time (mins)				
Glass	14	19	20	23
Silicone	30	32	34	38

The greater the diameter of the tubes the longer the clotting time in uncoated glass tubes In silicone coated tubes, clotting time is less influenced by increase in diameter of the tube

EXERCISE No 3 *Effect of pH on Clotting Time of Nonactivated Plasma*

Clotting Mixture 0 1 ml Imidazole Buffer (var pH)
0 1 ml Human Plasma
0 1 ml 0 02 M $CaCl_2$

Glass Tubes 38°C

Results

T be No	pH f Buff Solut	Cl tt g T m ( )
1	6 0	490
2	6 4	378
3	6 8	242
4	7 2	179
5	7 5	168
6	7 9	330
7	8 2	504

EXERCISE No 4 *Effect of Change in pH on the Clotting Time of Activated Plasma*

Clotting Mixture 0 1 ml Imidazole Buffer (variable pH)
0 1 ml Human Brain Thromboplastin
0 1 ml 0 02 M $CaCl_2$
0 1 ml Human Plasma

Glass tubes 38°C

The solutions of imidazole are prepared as described on page 11

was then introduced into each tube, recalcified as above and the clotting time measured

Results

Tube No	1	2
Surface	Glass	Silicone
Clotting Time (secs)		
Before rinsing	960	1495
After rinsing	601	1220

Repeated use of the same tube without proper processing tends to accelerate the clotting times of the plasma, especially when uncoated glass tubes are employed

EXERCISE NO 9 *Effect of Varying the Temperature on the Rate of Coagulation of Normal Platelet Poor Plasma*

Citrated plasma was placed in 13 mm wide silicone-coated tubes and kept in a constant temperature water bath for 3 minutes before recalcification

Clotting Mixture 0 1 ml 0 85% NaCl
0 1 ml Plasma
0 1 ml 0 02 M $CaCl_2$

Results

Tube No	1	2	3	4	5	6	7	8
Temperature (degree C)	20	25	30	35	38	40	45	50
Clotting Time (secs)	1440	1398	1020	775	560	588	875	1010

The optimum temperature for coagulation is near 38 C Temperatures of 35 or below or 45 or above delay clotting

EXERCISE NO 10 *Effect of Tilting Movements on Clotting Time in Glass and Silicone Tubes*

Two sets of 13 mm diameter tubes were arranged 5 glass and 5 silicone coated Eleven ml of blood were drawn and one ml placed in each of the ten tubes in a water bath at 38 C The clotting time of the blood in No 1 tube was determined After the blood in No 1 had coagulated the clotting time in No 2 was done etc The tubes were not disturbed until the blood in the preceding tube had coagulated

Rate of clotting was fastest in glass and slowest in silicone tubes

Clot retraction was greatest in silicone and paraffin, less in glass and absent in collodion

Reference

Lozner E L and Taylor F H L Foreign surfaces and blood coagulation J Clin Investig 21 241, 1942

Exercise No 7 *Effect of Using the Same Syringe on 2 Successive Determinations of the Venous Blood Clotting Time*

Blood was collected from a normal subject using a freshly coated siliconized syringe It was then placed in three 13 mm i d siliconized tubes *as indicated below, and the clotting time determined at 38°C* The syringe was then rinsed with 0 85 per cent NaCl several times, dried and used again as before Blood was collected from another vein of the same subject

Results

Tub N	1	2	3
Blood (ml)	1	0 6	0 3
0 85% NaCl (ml)	0	0 4	0 7
Per cent conc of the blood	100	60	30
Clotting Time (mins)			
(a) fresh syringe	34	22	32
(b) once used syringe	23	19	27

A significant shortening of the clotting time results when the same syringe is used for 2 determinations without recoating the surface of the syringe

Reference

Tulloch J A Overman R S and Wright I S Failure of ingestion of cream to affect blood coagulation Amer J Med 14 674 1953

Exercise No 8 *Effect of Using the Same Tube on 2 Successive Determinations of the Plasma Clotting Time*

Normal citrated human plasma was collected with the silicone technic 0 5 ml of the plasma was then pipetted into one uncoated and one silicone coated glass tube 13 mm i d 0 05 ml 0 2 M $CaCl_2$ was added to each and the clotting time measured at 38°C The clots were removed from the tubes 1 5 minutes after they were formed and then the tubes were rinsed 5 times with 0 85 per cent NaCl, inverted and allowed to dry 0 5 ml of the plasma

Clotting Mixture 0 1 ml 0.85% NaCl
0 1 ml Plasma
0 1 ml $CaCl_2$ (variable M concentration)

Results

Tube No.	1	2	3	4	5	6	7	8	9	10
Molar Conc $CaCl_2$	1	25	125	065	03	025	02	015	008	004
Clot Time (secs)	>5000	>5000	>5000	2160	392	390	317	482	>5000	>5000

As in the activated plasma clotting mixture a solution of 0 02 M con centration provides the optimum amount of $CaCl_2$ The nonactivated mixture is even more sensitive to excessive or insufficient recalcification than those containing thromboplastin Variations in hematocrit will there fore, be more promptly reflected in this than in the activated plasma clotting mixtures

Exercise No 13 *Effect of Varying the $CaCl_2$ Concentration on the Clotting Time of Activated Plasma*

$CaCl_2$ solutions of various molar concentration were prepared Using normal plasma and human brain thromboplastin the clotting time was measured in mixtures containing variable amounts of $CaCl_2$ The plasma was derived from blood to which trisodium citrate was added (1 part of a 3 8 per cent solution of citrate to 9 parts of blood) Since the hematocrit was 40 per cent 38 mg of citrate were contained in 6 ml of plasma or a citrate concentration of 0 63 per cent

Clotting Mixture 0 1 ml Thromboplastin
0 1 ml $CaCl_2$ (variable molar concentration)
0 1 ml Plasma

Temperature 38 C glass tubes

Results

Tube No	1	2	3	4	5	6	7	8	9	10
Molar Conc $CaCl_2$	1	25	125	065	03	025	02	015	008	004
Clotting Time (secs)	>5000	451	34	23	19	17	15	17	18	65

A 0 02 M solution of $CaCl_2$ supplies the optimal recalcification for plasmas with a citrate concentration such as that of the plasma tested If

Results

	Clotting Time in Seconds				
	Tube 1	Tube 2	Tube 3	Tube 4	Tube 5
Glass Tubes	400	600	750	900	1050
Silicone Tubes	775	1250	1450	1650	1850

Tilting the tube to detect the end point in clotting hastens coagulation in both glass and silicone coated tubes

EXERCISE No 11 *Effect of Multiple Venous Punctures on Clotting Time of Blood*

Blood was collected with the silicone technic from a normal person and a hemophilic patient with poor veins In the first venepuncture several attempts were made to obtain blood before the needle entered the vein In the second venepuncture another vein was easily found, the first few ml of blood discarded and blood for the clotting times collected with a fresh syringe Silicone tubes and syringes Temperature 38°C

Results

Tube No	Contents	Concentration of blood (per cent)	Clotting Times (min)			
			1st puncture		2nd puncture	
			Normal	Hemophil	Normal	Hemophil
1	1.0 ml blood	100	3½	8½	33	188
2	0.6 ml blood *0.4 ml 0.85% NaCl*	*60*	5	16	*18*	*150*
3	0.3 ml blood *0.7 ml 0.85% NaCl*	30	8	20	21	100

Repeated trial at *vein puncture* (1) *Accelerates* the coagulation of both normal and hemophilic blood especially the latter (2) Eliminates the clot accelerating effect produced by *dilution in both types of blood* (3) Also reduces or eliminates the difference between the rate of coagulation of blood in glass and silicone-coated surfaces (not *shown in table*)

EXERCISE No 12 *Effect of Varying the Concentrations of $CaCl_2$ on the Clotting Time of Nonactivated Plasma*

This exercise was performed with the same plasma used in the following exercise Instead of thromboplastin 0.85 per cent NaCl was used in the clotting mixture

REFERENCE

Tocantins L M Holburn R R Carroll R T and Stoker J W The rate of coagulation of the blood and plasma in contact with glass silicone and other surfaces Trans of the 3rd Conference on Blood Clotting of the Macy Foundation page 1?7 19.0

EXERCISE No 15 *Effect of Dilution on Clotting Time of Activated Plasma*

Same Plasma Used in Previous Exercise

Initial plasma concentration 98 per cent Temperature 38 C

Results

Tube No	Contents	Conc of Plasma (%)	Clotting time (secs.)	
			Silicone	Glass
1	0 5 ml Plasma 0 1 ml Thrombopl 0 05 ml 0 2 M $CaCl_2$	74	22	20
2	0 35 ml Plasma 0 165 ml 0 85% NaCl 0 1 ml Thrombopl 0 035 ml 0 2 M $CaCl_2$	52	20	19
3	0 16 ml Plasma 0 23 ml 0 85% NaCl 0 1 ml Thrombopl 0 16 ml 0 02 M $CaCl_2$	24	18	18
4	0 08 ml Plasma 0 39 ml 0 85% NaCl 0 1 ml Thrombopl 0 08 ml 0 02 M $CaCl_2$	1?	23	23
5	0 04 ml Plasma 0 47 ml 0 85% NaCl 0 1 ml Thrombopl 0 04 ml 0 02 M $CaCl_2$	6	42	42
6	0 02 ml Plasma 0 51 ml 0 85% NaCl 0 1 ml Thrombopl 0 02 ml 0 02 M $CaCl_2$	3	72	71

REFERENCE

Tocantins L M Carroll R T and Holburn R R Influence of the final plasma concentration in the actual clotting mixture on the response of normal and

a 3 8 per cent solution is used and the proportions of citrate solution to blood (1–9) are maintained, the optimum amount of calcium needed does not vary significantly provided the hematocrit is 40% ± 5 A high hematocrit will raise the citrate concentration of the plasmas while a low one will do the opposite, therefore altering the amount of $CaCl_2$ required for recalcification

EXERCISE NO 14 *Effect of Dilution on the Rate of Clotting of Plasma*

Fifteen ml of stable platelet poor normal plasma were separated from blood collected with a siliconized syringe containing 0 2 ml 19 per cent Na citrate per 10 ml blood The original plasma concentration taking 37 as the hematocrit would then be 98 per cent Temperature 38 C

Results

Tub No	Contents	Conc of Plasma (%)	Clotting Time (secs)	
			Glass	Silicone
1	0 5 ml Plasma 0 05 ml 0 2 M $CaCl_2$	89	360	1440
2	0 35 ml Plasma 0 165 ml 0 85% NaCl 0 035 ml 0 2 M $CaCl_2$	63	350	705
3	0 16 ml Plasma 0 23 ml 0 85% NaCl 0 16 ml 0 02 M $CaCl_2$	29	180	305
4	0 08 ml Plasma 0 39 ml 0 85% NaCl 0 08 ml 0 02 M $CaCl_2$	14	270	445
5	0 04 ml Plasma 0 47 ml 0 85% NaCl 0 04 ml 0 02 M $CaCl_2$	7	1040	1030
6	0 01 ml Plasma 0 53 ml 0 85% NaCl 0 01 ml 0 02 M $CaCl_2$	2	>3600	>3600

Dilution shortens the rate of coagulation of plasma and eventually equalizes the difference in the rate of clotting between plasma in glass and silicone-coated tubes

Results

Time of Centrifugation (mins)	15	30	60	120	240
Clotting Time (secs)	710	2650	8610	17,000	>20 000

Essentially incoagulable plasma may be obtained by sufficiently prolonged centrifugation provided an exacting technic is used in the collection separation, measuring and testing of the plasma

Exercise No 18 *Effect of Standing in a Plain and in a Silicone Coated Glass Vessel on the Rate of Coagulation of Plasma*

Ten ml of stable normal citrated plasma were collected with the silicone technic and 1 ml aspirated into each of the ten 1 ml pipets graduated in 0 01 ml Five of the pipets were of plain glass and five were of silicone-coated glass The filled pipets were allowed to stand at room temperature (18–20°C) for the periods of time indicated below before testing plasma at 38 C in silicone-coated tubes

Clotting Mixture 0 5 ml plasma
0 05 ml 0 2 M $CaCl_2$

Results

Duration f Standing (mins)	0	5	15	30	60
Clotting Time (secs)					
Glass	2250	1510	1365	1150	410
Silicone	2280	21 0	2270	2310	2270

The clotting time of plasma is markedly shortened by short periods of standing in uncoated glass vessels but little affected when in contact with silicone coated surfaces

Reference

Tocantins L M Influence of the contacting surface on the coagulability and anti cephalin activity of normal and hemophilic plasmas Am J Physiol *143* 67–76 1945

Exercise No 19 *Effect of Tissue Thromboplastins from Different Species on Rate of Coagulation of Plasma*

Materials (1) Citrated plasma from rabbit mouse and man (2) Thromboplastin prepared by the method described on page 101 using acetone dried (a) human (b) rabbit and (c) mouse brain

hemophilic plasmas to thromboplastin Trans of the 3rd Conference on Blood Clotting and Allied Problems of the J Macy Jr Foundation page 192, 1950

EXERCISE NO 16 *Effect of Centrifugation of Normal Blood on Rate of Clotting of Plasma*

Fifty ml of citrated blood collected with the usual precautions were centrifuged at 1000 rpm for various lengths of time as listed below When a sample was to be taken 1 5 ml of plasma from the upper portion was removed by a capillary dropper and placed into a separate tube The remaining sample was centrifuged again for the periods stated below Care was taken *not to disturb the buffy layer when aspirating the plasma*

Clotting Mixture 0 5 ml plasma
0 05 ml 0 2 M $CaCl_2$

Results

Duration of Centrifugation (mins)	Total Centrifugation (min)	Clotting Time (secs)
8	8	960
7	15	1030
10	25	1140
20	45	1590
45	90	2100
90	180	2300

When testing samples in duplicate or triplicate the three tubes are held at once and tilted at the same time to detect the end point of clotting

If a platelet count is done on each plasma sample a relationship will be seen to exist between the number of platelets and the duration of the centrifugation *The longer and faster the centrifugation (the greater the g) the* less platelets will be in the supernatant plasma and the longer the clotting time More prolonged centrifugation will in addition result in displacing lipid anticoagulant upwards and further prolonging clotting time

EXERCISE NO 17 *Effect of Centrifugation on Rate of Clotting of Normal Plasma*

Twenty ml of platelet poor normal plasma were separated from properly collected blood centrifuged in silicone coated tubes for ½ hour at 1000 rpm The plasma was divided into five tubes and each of the tubes was centrifuged at 5 C in a horizontal head for the periods stated below The upper half of each plasma specimen was then removed and tested Silicone surfaces 38 C

Clotting Mixture *0 5 ml* plasma
0 05 ml 0 2 M $CaCl_2$

EXERCISE No 21 *Effect on the Clotting Time of Varying the Concentration of the Plasma but Maintaining that of Thromboplastin and $CaCl_2$ Constant*

Normal and hemophilic human citrated plasma collected with especial precautions Human Brain Thromboplastin as activating agent
13 mm i d Silicone Tubes 38 C

Results

Clott g Mixture	T tal V lum ml.	Plasma Co %	Throm bopl. Conc ing %	Clott g T'm (secs.)	
				N rmal Plasm	Hemo- ph lic Plasm
0 1 ml Plasma 0 1 ml 1-10 dilut T plastin 0 1 ml 0 02 M $CaCl_2$	0 3	30	30	26	27
0 4 ml Plasma 0 1 ml 1-5 dilut T plastin 0 1 ml 0 08 M $CaCl_2$	0 6	59	30	28	30
1 3 ml Plasma 0 1 ml 1-2 dilut T plastin 0 1 ml 0 ^6 M $CaCl_2$	1 5	77	30	32	44
2 8 ml Plasma 0 1 ml undil T plastin 0 1 ml 0 56 M $CaCl_2$	3 0	83	30	35	48

High concentrations of plasma delay clotting of activated normal plasma and even more that of hemophilic plasma

Clotting Mixture 0 1 ml plasma
0 1 ml thromboplastin
0 1 ml 0 02 M $CaCl_2$

Results

Source of Thromboplastin	Clotting Time (Seconds)		
	Human Plasma	Rabbit Plasma	Mouse Plasma
Human brain	12	14	16
Rabbit brain	12	7	13
Mouse brain	72	22	9

Each thromboplastin seems to be most effective when tested on homologous plasma

EXERCISE No 20 *Effect of Addition of Thromboplastin of Variable Concentration on Clotting Time of Plasma*

Clotting Mixture 0 1 ml Human Brain Thromboplastin (variable dilution)
0 1 ml 0 02 M $CaCl_2$
0 1 ml Human citrated plasma

In this mixture the plasma concentration is about 26 per cent

Dilution of the thromboplastin made with 0 85 per cent NaCl immediately before use

Results

Tube No	Concentration of Thromboplastin (per cent)	Clotting Time (secs)
1	100	21
2	50	22
3	25	22
4	12 5	25
5	10	25
6	5	30
7	2 5	34
8	1 0	78
9	0 5	120
10	0 25	190
11	0 125	254

nificant differences in certain components of the clotting system These may be advantages or disadvantages, depending on the problem

MEASUREMENT OF THE CLOTTING TIME

There seem to be more methods than investigators in this regard The essential point is standardization of temperature, container and observation Clotting is a gradual process Also it is a general experience that the clotting time is related exponentially with the concentration of any factor In selecting an end point if the final change from unclotted to clotted takes one half minute for a 4½ minute clotting time it will take 2½ minutes for a 22½ minute clotting time Likewise when variations in thromboplastin in the blood sample due to variations in trauma of the vein give a normal clotting time of 4 ± 1 minute this means a clotting time of 20 minutes will vary between 15 and 25 minutes Attempts to improve the reading of the end point cannot alter this situation Mechanical devices are useful in allowing *one observer to handle multiple samples They must not be relied on to* improve accuracy or replace continued critical judgment Both 37 C and room temperature (15, 20 or 25 C) have been used The previous remarks on selection of method apply equally to selection of temperature In all cases it is essential that sufficient determinations can be done to obtain means with significant standard deviations For normal values this must be done frequently It is not unusual to find that over a period of time with continuous practice the mean normal value obtained by one observer or for one laboratory will change Normal values may be found elsewhere [1] For the present purpose of greater significance is the standard deviation (s d) expressed as a per cent of the mean for a wide range of value of means For the Lee and White method values of the mean range from 5 to 60 minutes

Three common methods are described These are sufficient for most purposes The selection of one of these or a combination of them should be made in the light of the above principles

Lee and White Method[2]

Materials Glassware needle etc must be scrupulously clean Five ml syringe rinsed with normal saline solution immediately before use 18 or 20 gauge steel needle 8 mm test tubes washed with soap water chromate cleaning solution rinsed well with water and dried Test tube rack Water bath at 25 C or 37 C (A pan of water with thermometer and adjusted with tap water just before use is sufficient in ordinary circumstances A thermos jar is useful for bedside work) Stopwatch

Procedure After entering the vein withdraw 3 ml of blood Discard 1 ml and transfer the second ml to a 8 mm test tube in water bath Tilt

CHAPTER III

DETERMINATION OF THE CLOTTING TIME OF WHOLE BLOOD

L B JAQUES

Object The measurement of the rate of clotting of blood after removal from the body

Warning The determination of clotting time is the *simplest*, the *most informative*, and technically the *most difficult* of all common laboratory procedures, further continual practice with alert attention to all details is essential The multiplicity of techniques and undeserved censure of the method can be attributed to failure to appreciate this

The test consists of two parts (a) collection of the blood sample and (b) measurement of the clotting time Each of these steps must be carefully standardized

Collection of the Blood Sample

From the standpoint of the sources of error and the significance of the test this is the more important part of the test The blood sample may be (a) venous blood taken with glass syringes (b) cutaneous blood (c) venous blood taken with silicone (plastics) coated apparatus The selection of methods is determined by the problem If it is necessary to detect a shortening of the clotting time then a method yielding a long clotting time on normal blood will enhance the possibility of doing this If however a quantitative measurement is being sought of blood in a state of extreme hypocoagulability then a method which will give shorter values in normal blood may be more desirable In addition selection of the method may be dictated by the *qualitative* nature of the change in blood coagulability under study In general the clotting time of cutaneous blood is less sensitive to changes in composition of the clotting system Variable admixture with tissue juices from the puncture site is a drawback and may seriously impair the usefulness of this source of blood sampling With venous blood the reactions of the clotting system induced by contact with a glass syringe will mask sig

never so rapidly that the vein is deflated or turbulent flow occurs in the syringe Removal of blood should balance the rate of entry of blood into the vein Conditions such as shock offer a problem in determining clotting times Very slow removal with a siliconized syringe is probably best for this In taking repeated samples care must be taken not to use the same site With exposed veins we have found that 1 cm is the closest that a second puncture should be

Determination

Sources of error here are variations in the surface of the tube, i e, degree of cleanliness (see page 7) variations in number of times the tube is tilted and degree of tilting determination of the end point The latter requires experience It should be pointed out that the end point is quite different to that used in estimating the clotting of plasma It is equally reproducible

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the tube at intervals of fifteen to thirty seconds until clotting occurs At this time the tube can be turned upside down

Range 2 s d as per cent of mean = ±73 per cent

Capillary Tube Method[3] [8]

Materials Five capillary tubes with lumen of circa 1 mm length of 5 cm, vaseline, alcohol, automatic lancet Stopwatch

Procedure Clean tip of finger with alcohol and cover with vaseline Noting the time, make a deep needle puncture so that blood flows *freely* without milking' Discard first two drops of blood, then allow tubes to draw up the following drops Fill only ¾ of tube At 30 second intervals tilt tube and notice flow of blood column When this ceases break off short sections of tube every thirty seconds until a fibrin thread spans the gap between the fragments

Range 3–5 minutes

Silicone Surface Method[4] [5]

Materials 5 ml glass syringe treated with silicone Dri Film 9977 or 9987 (see page 3) 18 or 20 gauge needle treated with Arquad 2c (see page 6) 8 mm test tubes treated with silicone, test tube rack Water bath at 25 or 38°C (pan of water with thermometer), stopwatch A thermos jar with water at temperature desired may be used at bedside

Procedure 5 ml of blood are taken *carefully* using the silicone syringe and needle The needle is removed and 1 ml of blood discarded Three one ml samples of blood are placed in 3 siliconized test tubes in the water bath and the remainder of the blood discarded If not advisable to take 5 ml 3½ ml may be used provided the middle portion is used The tube (s) is tilted carefully at intervals until the blood no longer flows The forming of a round surface in contrast to liquid flow provides a definite end point

Range 2 s d as per cent of mean = ±25 per cent

Sources of Error in Blood Clotting Time Determinations

Collection of the Venous Blood Sample

Most of the variations in values can be traced to this source The syringe must be a good fit likewise the needle and both be scrupulously clean There must be a good blood flow The needle must enter the lumen cleanly and must not be caught in the vein wall It must remain in the lumen If it catches on endothelium or valve leaflet the clotting time will be greatly reduced After centering the needle in the vein a pause of 2–3 seconds for blood to remove traces of tissue from the end of the needle is advisable The blood is then drawn as rapidly as possible into the syringe but

Apparatus (fig 1) A tapered glass tube (A) closed by a cork stopper on the bottom to which a gnarled glass rod has been attached (B) the inside surface of the tube and the cork bottom are coated with paraffin (M P 50 C) but the glass rod is left bare When these are assembled they appear as in (C) A graduated tube (D) like that used for the Addis urine count technic

Steps in the Procedure Collect 2 ml of venous blood and place exactly 1 0 ml in the assembled tube which is then stood up in the incubator at 38 C The rest of the blood is used to determine the per cent packed cell volume (hematocrit) Determine the clotting time of the blood in the tube by tilting it at intervals When clotting is complete allow one hour to elapse then invert the small tube and insert it into the graduated tube as shown in (E) (fig 1) Read the volume of the serum collected at the graduated stem of the tube Reexamine in another hour Then remove the gradu

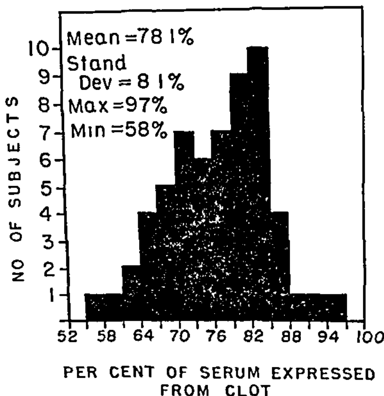


Fig 2 —Retraction (2 hours) of clots from 1 cc of venous blood in a paraffin tube 13 mm i d at 37 C (59 adults)

CHAPTER IV

MEASUREMENT OF THE RATE AND EXTENT OF CLOT RETRACTION

L M TOCANTINS

Object A quantitative estimation of how much and how fast a clot retracts after it has been formed

Principle The method is essentially like that of Adreassen Blood is allowed to clot in a paraffin coated vessel in the center of which is an uncoated glass rod The blood adheres to the glass but not to the paraffin thereby making it possible to invert the vessel and measure the expressed serum while the clot remains adherent to the glass

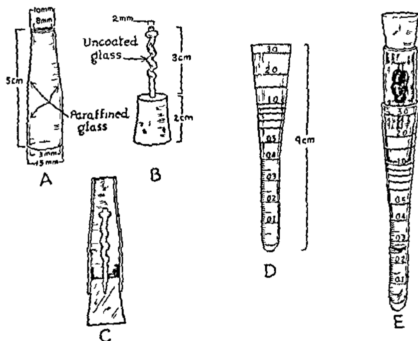


FIG 1 —Apparatus

CHAPTER V

BLOOD PLATELETS

1 Counting Platelets in the Blood

L M TOCANTINS

Most technical methods for counting platelets utilize either cutaneous or venous blood The choice of methods obviously must depend to a certain extent on the experience of the one who is to use the method For *inexperienced* workers the order of choice should be (1) indirect platelet count on cutaneous blood with an isotonic solution containing a stain a fixative and an anticoagulant (2) indirect count on venous blood (3) direct platelet count on cutaneous blood (4) direct platelet count on venous blood The ratio of erythrocytes to platelets should be established from at least 1 000 erythrocytes The choice of methods is further conditioned somewhat by the type of blood to be examined With leukemic and thrombopenic blood counting on wet specimens should be supplemented by a method in which the dry stained smear is used such as Fonio s as a further check on the number of platelets or as a means of avoiding the inclusion of any hyaline platelet like bodies in the total count Venous blood is preferable to cutaneous blood The trauma involved in an incision of the skin is greater than that in a venous puncture with a small needle (from 26 to 27 gauge) When one considers the role played by the platelets in repairing the effects of trauma it is logical to anticipate that the trauma involved in an incision of the skin will affect the number of the platelets Comparative determinations of the platelets in vessels with and without trauma yield great differences and this fact renders serial counts from the same location within short periods of time of questionable value Appreciable numbers of platelets are lost in the lips of the wound before the blood reaches the surface With a technic such as that of Tocantins or Preiss[4] the blood passes from the lumen of the vessel directly into the lumen of the needle where it is immediately diluted and mixed with the anticoagulant and (preferably) fixed all surfaces of contact having previously been wetted by the solution Counts done on venous blood are more representative of the actual conditions in the circulation for a larger amount of blood is usually collected for examination than when a cutaneous puncture is used It is also possible to repeat

ated tube and centrifuge for 15 minutes at 2000 rpm Read the level of packed cells and the expressed serum

Calculation Results are expressed in terms of the percentage amount of serum expressed from the clot at the end of 2 hours taking into consideration the amount of potential serum present in the clot (calculated from the hematocrit determination) *Example* 1 0 ml of blood with a percent cell volume of 46 expressed 0 4 ml of serum at the end of 2 hours

$$\text{Percent serum expressed} = \frac{0\,4 \times 100}{0\,54} = 74\%$$

0 54 ml is the total amount of serum potentially present in 1 0 ml of the specimen of blood

Range of Values Mean of 59 determinations in 59 adult men and women 78 1 per cent (Stand Dev 8 1 per cent), range 97–58 per cent The frequency distribution is shown in figure 2

Precautions and Sources of Error The glass rod must be left uncoated or the clot will not adhere to it When the rate of clotting is unusually slow or imperfect (as in hemophilic blood) the method is unreliable since partial coagulation renders the clot nonadherent and easily displaced The "serum" expressed is in reality only partly serum

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platelets in plasma during the time required for sedimentation of the erythrocytes

As anticoagulants sodium citrate sodium oxalate sodium metaphosphate sodium or magnesium sulfate heparin peptone and gelatin have been used in diluting solutions Of these sodium citrate seems to be the best Oxalates often form crystals and precipitates with the plasma Sodium metaphosphate should be in higher concentrations (from 5 to 10 per cent) than those generally used (2 per cent) Heparin solutions are difficult to make clear even after prolonged centrifugation Peptone and gelatin like sucrose and dextrose encourage the growth of bacteria None of these anticoagulants prevents morphologic changes in the platelets without the addition of a fixing reagent The preservation of the morphologic character of the platelet for total and differential counting may be obtained only by the dual process of simultaneous prevention of coagulation of the blood and rapid fixation Good fixation depends not so much on a high concentration of the fixative as on the promptness with which even a weak fixative comes in contact with the platelets after these have left the vessels As fixing reagents mercury bichloride 1 and 2 per cent osmic acid solution of formaldehyde U.S.P methyl and ethyl alcohol and potassium bichromate have been used Of these solution of formaldehyde U S P in a concentration of about 0 1 per cent seems the best Diluting fluids containing this solution of formaldehyde in percentages of 2 and 2 5 per cent often hemolyze erythrocytes and form precipitates with the plasma It is important to use neutral solution of formaldehyde in the preparation of solutions for if enough formic acid is present it will hemolyze the red blood cells When a solution containing formaldehyde has stood for a time some of the formaldehyde is oxidized and formic acid is formed When this acid reaches a concentration of from 0 05 to 0 075 per cent it decolorizes the erythrocytes even in isotonic salt solution although it does not affect platelets or leukocytes The hemolysis that is occasionally encountered when one is using the Rees Ecker solution is chiefly due to this When a diluting fluid containing formaldehyde begins to hemolyze the red blood cells it should be discarded and the glassware that has been in contact with it should be thoroughly cleaned Methyl and ethyl alcohol are poor fixatives for platelets in wet preparations for they precipitate the plasma proteins and cause secondary changes in the platelets Mercury bichloride in a concentration such as that in Hayem solution B (0 25 per cent) seems a good fixative In higher concentration it often produces protein precipitates Osmic acid while an excellent fixative for dry smears is expensive and often produces clumping of erythrocytes in wet preparations Approximately half of the diluting solutions proposed do not include a fixative

The inclusion of a stain in the diluting fluid is not necessary except for

the count and make observations on the other morphologic constituents of the blood

For greater accuracy, in experienced hands, direct methods are preferable to indirect ones *Most of the objections usually raised against the use* of the direct method apply only if it is employed by one who is inexperienced with the manipulative procedures involved Even on cutaneous blood, if there are free bleeding from the wound, rapid coordination of movement, use of scrupulously clean glassware and solutions, duplicate determinations and uniform shaking this method is useful

Some of the objections to the indirect method of counting are The mixture of blood and diluting solution is seldom, if ever, uniform and not the same each time The ratio of erythrocytes to platelets in a given sample of blood determined on the counting chamber often varies widely from that determined in wet smears Platelets and erythrocytes are not distributed evenly through the preparation since no provision is made for shaking before counting It is obvious that the greater the number of erythrocytes and platelets counted the more accurate will be the ratio established between these two elements if a counting chamber is used to establish this ratio, the total number of erythrocytes counted will vary depending on the dilution employed The method has defects intrinsic in any determination done indirectly that is in relation to another equally changeable element The greatest source of error however is in the assumption that platelets and erythrocytes keep an even proportion in numbers toward each other between the two main steps of the method The markedly different physical properties (adhesiveness, specific gravity size and others) of platelets and erythrocytes lead to continuous changes in this ratio Finally the proportion of platelets to erythrocytes varies at the same time in different portions of the circulation and this variation is even more marked in capillaries and venules

With the Thomsen[2] method and its modifications, the platelets are counted in the plasma free from other cells This is advantageous in some respects it decreases the possibility of confusion with the products of the *disintegration of erythrocytes* besides having all the other advantages of methods in which venous blood is utilized Although the Thomsen method at first seems a direct method of counting platelets in whole blood, it actually is an indirect method for one must always have a supplementary determination of the volume of packed cells or of the hemoglobin to calculate the absolute number of the platelets per cubic millimeter of blood This multiplicity of determinations may contribute to increase the error but unlike the indirect method in which use is made of the erythrocyte platelet ratio the determinations are all done in the same sample of blood There is also some question as to the stability and uniformity of the suspension of

(depth 0 1 mm) and the Thoma diluting pipets if properly calibrated and certified seem entirely adequate

A magnification of from ×400 to ×600 with dry objectives seems best Higher magnifications may be obtained with chambers less than 0 1 mm in depth and with use of thin cover glasses Oil immersion objectives often disturb the cubic content of the chamber and the use of low power objectives is not advisable While identifying platelets the observer should have the fingers of one hand on the fine adjustment to obtain the critical focusing that reveals the characteristic highly refractile silver chip appearance of the platelet Only forms from 1 to 3 microns or longer in size rod or comma like if seen sidewards and thin translucent and disklike if flat on the floor of the counting chamber should be counted Granules 0 8 micron in diamter or smaller jerkily moving about more or less actively globules of oil irregularly shaped debris floating on the upper layers of the fluid strings of cocci and other minute objects may be distinguished from platelets after a little practice The error of counting too few platelets may be equaled only by the error of counting every particle in the field as a platelet

Many of the suggested precautions are often useful under certain con ditions Platelets may be counted however just as easily as erythrocytes if coagulation of the blood is effectively prevented and the platelets are fixed as the blood is withdrawn without contact with the tissues or any wet table surfaces It is the initial stage of alteration in the platelet that renders it so strongly adhesive and agglutinable Rapidly fixed platelets preserve their size and shape and adhere very little to glass surfaces or to each other Therefore a diluting fluid for total and differential platelet counting should (1) effectively prevent coagulation of the blood and changes in the platelets (2) fix the platelets as the blood is being removed from the vessels (3) be isotonic with the blood and cause no precipitates with the plasma or the glassware and (4) be of simple composition and of easy preservation Of the fluids available for platelet counting the Rees Ecker solution (of which the solution to be described is only a slight modification) seems to have all the necessary ingredients in the best combination A stain is necessary only until one has gained experience in recognizing platelets The following solution has been found to fulfill the requirements sodium citrate 3 8 Gm solution of formaldehyde (neutral 40 per cent) 0 2 ml distilled water 100 ml If a stain is desired add 0 05 Gm of brilliant cresyl blue Keep in a well stoppered bottle in an icebox filter each time just before using per form blank counts on the solution at intervals and look for bacteria molds and platelet like bodies Use glass stoppers for all containers The use of cotton or lint should be avoided

beginners or in certain pathologic conditions Brilliant cresyl blue, methyl violet, methyl green, neutral red, methylene blue and eosin have been used Brilliant cresyl blue in a concentration of from 0 05 to 0 025 Gm per hundred cubic centimeters seems the best Methyl violet is satisfactory while the others, like methyl green stain the platelets poorly or not at all For counting on dry smears, the Giemsa or Wright stain demonstrates the platelets best Stains aid the reading of the meniscus in pipets and syringes but increase the chances of producing artefacts and the labor of cleaning glassware

The use of diluting solutions that hemolyze the red corpuscles is generally inadvisable The erythrocyte count which should accompany every platelet count is made impossible many artefacts are created and it is difficult to be sure that the platelets themselves are not sometimes affected In experienced hands however these solutions may be used advantageously The hemolyzing agents employed have been potassium cyanide and urea

Diluting solutions resembling the plasma in composition, such as Tyrode and Ringer solution, offer no especial advantage in the counting and morphologic study of intact platelets Their similarity to plasma in composition may be a handicap in this study for there are few fluids in which platelets disintegrate more rapidly than in plasma collected without precautions to prevent its coagulation Adding a fixative such as mercury bichloride, to Tyrode solution remedies the disadvantages of this solution partially but such an addition obviously alters its delicate chemical balance thus defeating the original purpose in using it and making its use no more advantageous than that of other far simpler solutions Both Tyrode and Ringer solution encourage the growth of bacteria neither keeps well and the multiplicity of chemical substances in each is a constant source of artefacts Methods in which Tyrode diluting fluid is utilized invariably yield high platelet counts on cutaneous blood The platelets observed in excess over the usually accepted normal number are however of the micro' type and it has been demonstrated that most of these forms are artefacts formed either by contact of the blood with tissue juices in the presence of Tyrode solution or by precipitation of the calcium chloride of the solution when this is in contact with the alkali of the glass

Pipets and syringes of special shape and composition have been employed although useful in the hands of their originators they offer little advantage over the standard types available Counting chambers such as that of Helber (depth 0 02 mm cover glass thickness 0 1 mm) the Petroff Hausser counting chamber for bacteria (depth 0 02 mm cover glass thickness 0 18 mm) are useful but do not seem strictly necessary The standard Burker Thoma Zeiss and Levy Hausser counting chambers

same time on blood from various channels are done preferably in this order (1) venous blood (2) arterial blood (3) cutaneous blood Whenever the syringe is used for making dilutions it is essential for the operator to place himself and so arrange the arm of the subject that the syringe is held horizontally This insures easy reading of the marks on the syringe without making errors because of parallax and insures detection of the moment when the artery is entered for the pressure in the vessel will overcome the column of solution in the syringe if the latter is held horizontally The syringe should be so turned that the graduation lines are on each side of, and not over the piston Measurements are made by adjusting the end of the piston along the same line as graduation markings on the side thus preventing parallactic errors which are unavoidable when the end of the piston is adjusted under the ruling

If the platelets are above 1 000 000 per cubic millimeter dilutions higher than 1 5 are used to reduce the chances of agglutination The contention that high dilutions of blood yield correspondingly higher counts because of fragmentation of platelets applies only when no fixatives are used If there is reason to expect a low platelet count the blood is diluted 1 2 in the syringe instead of 1 5 and special precautions should be taken with the diluting fluids If the platelets in the chamber are few the presence of any extraneous matter in the diluting solution will often cause the most experienced technician to include them in the count if they resemble platelets The most scrupulous cleaning of the glassware and filtering of the solutions will leave a very small amount of particles in suspension When the platelet count is normal these particles usually do not greatly alter the result since in any solution they should not be present in greater concentration than 5 particles per tenth of a cubic millimeter of the solution When few or no platelets are present however they may look enough like platelets to cause the observer to count them as such thereby greatly exaggerating the platelet count If the diluting solution includes a dye like brilliant cresyl blue this difficulty is increased For this reason with every series of platelet counts a blank count on the filtered solutions should always be done and the result subtracted from the platelet count if the solution contains only 5 or less platelet like bodies per tenth of a cubic millimeter This practice not only increases the accuracy of the count but gives also a frequent check up on the solutions The total number of platelets is calculated by subtracting the number of platelets counted in the blood dilution preparation (a) from the number of platelet like bodies per tenth of a cubic millimeter in the diluting solution (b) according to this formula

$$\frac{(a - b) \times \text{dilution} \times 4\,000}{400} = \text{number of platelets per cubic millimeter of blood}$$

Method for Counting Platelets in Venous Blood

Apparatus a syringe of the type used in making the tuberculin tests, having a capacity of 0 5 ml and graduated in tenths or five hundredths of a milliliter, vials of 2 ml capacity, containing a glass bead and having glass stoppers ground to fit, an automatic shaker, pipet and counting chambers certified by the United States Bureau of Standards The solution is kept at from 2 to 4 C after being filtered It is placed in small test tubes and sterilized in the autoclave along with tubes containing distilled water, loss in fluid in the solution during sterilization being made up with the distilled water The needles, syringes and vials are sterilized by dry heat Counting chambers and cover slips are boiled once weekly for two hours in distilled water containing a small amount of sodium bicarbonate pipets are cleaned by the usual changes of water, ether and alcohol

Steps in the Procedure Aspirate 0 4 ml of the diluting fluid into the syringe and dry the tip with a sterile gauze stopper Adjust a sterile No 27 gauge needle to the tip snugly to prevent any ingress of air Then carefully push the solution up the shaft of the needle until it just appears at the lumen For needles of the size specified 0 03 ml of solution is usually needed to fill the shaft of the needle This therefore will displace the piston downward Transfer to the sterile vial enough of the solution to bring the piston down to coincide with one of the rulings on the barrel Enter the vein after distending it slightly by application of a little pressure above the site of puncture Once the needle is in the vein remove the pressure, and allow approximately five seconds to elapse before withdrawing blood Slowly withdraw exactly 0 1 ml of blood into the syringe Then place the tip of the needle under the level of the solution in the vial and empty the contents of the syringe into it, aspirating and emptying the solution and blood slowly, without bubbling three or four times to insure thorough mixing Stopper the vial and place it in an automatic shaker where it is to be rhythmically agitated for at least five minutes From the shaken mixture make a 1 100 dilution using a diluting pipet and the same diluting fluid Submit this dilution also to shaking then place a drop of it in two counting chambers and count the erythrocytes in 160 of the small squares of each chamber multiply the average of the two counts by 12 500 Allow the preparation to stand in a moist chamber for at least twenty minutes before counting the platelets *Count all platelets in 400 small squares (or the entire finely ruled area of each chamber)* and multiply the average of the two counts by 5 000

In man, the basilic veins in the forearm are used for venous platelet counts in dogs, the leg arm or ear veins and in rabbits the ear veins For platelet counts on arterial blood in man the radial artery is the most accessible, the brachial artery, at the elbow may also be used in dogs the femoral artery along the thigh is the most accessible Counts done at the

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2 Estimation of the Number of Platelets by Phase Microscopy

GEORGE BRECHER and E P CRONKITE

The objectives of any counting method for cellular blood elements are twofold To obtain a representative sample and to estimate without bias the number of cells in that sample

Avoidance of adhesion of platelets to needles and glassware during collection and dilution is the main problem in obtaining a representative sample This requirement is best met by use of venous blood obtained with a 19 or 20 gauge needle with minimum of trauma and with rapid dilution of the sample

The objective of obtaining an unbiased estimate of the number of platelets is best met by the use of customary dilution pipets and counting chambers The small size of the platelets makes their recognition in the counting chamber with ordinary microscopy moderately difficult except for experienced investigators Indirect methods have therefore been widely used The realization that platelets are readily recognized in the counting chamber by phase microscopy has removed older objections to the direct method Direct platelet counts under the phase microscope are therefore considered the method of choice Other direct methods (Rees Ecker Tocantins)[8] are also satisfactory but require considerable skill in the actual recognition of platelets in the chamber

The touchstone of any method for the counting of platelets is its reproducibility and lack of bias Simple duplication of results on the same sample is inadequate as proof of reproducibility and lack of bias of the count The proper statistical treatment[1 2] of data to check reproducibility and lack of bias of any direct or indirect method is considered the sole minimum requirement for platelet counts[3 4]

The use of the phase microscope for platelet counts was suggested by Feissly[5] and more fully developed by Brecher and Cronkite[3 4] The following instructions are given from the paper by Brecher Schneiderman and Cronkite[4]

Apparatus Required Flat bottom counting chamber Long working distance phase condenser with 43X annulus and matching 43X phase

In thrombopenic bloods platelets are further searched for in a smear of blood made from the mixture and stained with Wright or Jenner Giemsa stain, after the erythrocytes have settled in the vial, a loopful of the super natant plasma is examined for the presence of platelets as a hanging drop Occasionally (when the count is very low) the drop of plasma may be introduced without further dilution into the counting chamber, the platelets in 400 small squares counted, and the total number of platelets per cubic millimeter of plasma obtained by multiplying the total number counted by 10

Values found Mean of 40 determinations on 40 normal adult men 310 000 per cu. mm (Max 690,000 Min 150,000)

Method for Counting Platelets in Cutaneous Blood

Rees-Ecker Direct Method Aspirate the following freshly filtered solution up to point 0.5 of a pipet for counting erythrocytes Sodium citrate 3 8 Gm 40 per cent formaldehyde solution 0 2 ml brilliant cresyl blue 0 1 Gm and distilled water 100 ml Make a puncture 3–4 mm deep in the finger and from the freely bleeding wound, aspirate enough blood to push column up to point 1 Fill the remainder of the pipet with the solution up to 101 Shake place the 4th or 5th drop in the counting chamber (Levy Hausser) wait for 15–20 minutes for settling and then count the platelets in the entire finely ruled area (400 squares) Multiply the total by 2 000 to obtain the number of platelets per cu mm of blood

Values Found Mean platelet count in 40 young adults 250,000 Range (2x stand dev) 243 000–257,000

Indirect Method on Dried Stained Blood Smears (Modified Fonio) Place a large drop of 14 per cent magnesium sulfate solution over the skin of the finger which must be clean and dry Puncture the skin through the liquid, collect a drop of the blood liquid mixture with a paraffin coated glass rod place it on a clean slide and make a smear Fix the smear in absolute methyl alcohol (two minutes) and stain for from three to five minutes with Wright s stain (10 drops of stock Wright s solution added to 10 drops of Wright buffer) Wash with distilled water dry and examine with the oil immersion lens Establish the ratio of platelets to erythrocytes from at least 1 000 erythrocytes Calculate the number of platelets from this ratio and an erythrocyte count done at the same time

Values found Mean platelet count in 30 male adults 234 000 Range Max 350 000 Min 130 000

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clumps indicates that the dilution of the blood with diluent has been delayed too long and a fresh sample must be taken This occurs but rarely On focusing up and down platelets can be seen to have one or more fine processes Crystals dirt and bacteria are readily distinguished by their refractility and absence of pink purple sheen With even slight experience it is not necessary to visualize the processes of individual platelets and the count can be completed quickly, once the platelets have settled out without more than occasional refocusing

The optimal procedure of recording counts is to write down the count for each of the 10 blocks of small squares in each chamber This allows computation of the field error due to chance distribution of the cells in the chamber With this method the error of a single platelet count is 11 per cent When 8 pipets and chambers are used on a single sample the error is slightly less than 4 per cent These are the minimum errors due to pipet and chamber tolerances allowed by the National Bureau of Standards and the chance distribution of cells in the chambers (when 50–150 cells are *actually counted per chamber)*

When platelet levels are very low, more than the standard 10 blocks of small squares must be counted if the errors are to be kept within the limits given in the preceding paragraph This can be accomplished either by using lower dilutions (1 20 or even 1 10 using WBC instead of RBC pipets) or by counting all 25 blocks of small squares in each half of the chamber The latter is usually preferable because with very low dilutions of blood the individual platelets are not quite as readily recognized in the chamber due to increased RBC detritus In either case the calculation must be appropriately adjusted e g when 25 blocks of small squares are counted on each side of a chamber or a total of 50 the result is multiplied by 500 (rather than 2 500) to give the number of platelets per cu mm

Range of Values The mean normal platelet level of healthy adult American and British males is about 250 000/cu mm There is however marked variation between individuals Ninety five per cent of healthy males have counts between 140 000 and 440 000 In any given individual the platelet level stays usually constant over many days months or even years In females platelet levels are probably similar but variations with menstruation have been reported Indirect counts have generally given higher values but these are believed to be due to selection of areas counted (e g central areas of wet smears) and not due to actually higher circulating platelet levels[7, 8]

Precautions and Sources of Error The counting chamber should be scanned for presence of platelet clumps which indicate undue delay between collection and dilution of the sample The presence of platelet clumps precludes reliable counts and a fresh sample must be collected

objective (If using American Optical equipment specify 'medium dark contrast') RBC dilution pipets (for 1 100 dilution) Pipet rotor Siliconed Kahn test tubes

Solution Required One per cent ammonium oxalate in distilled water (keep in refrigerator when not in use to avoid growth of bacteria or preferably keep stock bottle always in refrigerator, remove a few ml for day's work as needed and discard at end of day)

Steps in Performance of Method (1) Venous blood is collected by inserting a 20 gauge needle, without syringe, into a cubital vein, and by allowing 1–2 ml of blood to flow directly into a siliconed test tube The siliconed test tube is kept in a beaker with ice water, both before and after the collection of blood Immediately after collection the blood was diluted in red blood cell pipets An alternate method is to collect the blood with a 19 or 20 gauge needle and a clean siliconed syringe, with a minimum of suction to avoid air bubbles After removing the needle, the blood is gently expelled into a siliconed test tube If only single or duplicate counts are contemplated the cooling of the test tube in ice water may be omitted Regardless of the method of collection, the blood must be diluted in the red blood cell pipets as quickly as possible

(2) The blood is drawn up to the 1 mark of a red blood cell pipet and 1 per cent ammonium oxalate to the 101 mark, giving a 1 100 dilution This step must be completed without delay Subsequently the pipets are kept rotating in one of the commercially available pipet rotors until the chambers can be conveniently filled and counted Rotation of pipets for as long as 8 hours does not affect the counts

(3) A hemocytometer is filled in the usual fashion, except that a No 1 or 1½ cover slip* is used rather than the standard hemocytometer cover glass of 0 4 to 0 6 mm thickness The use of a thin cover slip is essential in phase microscopy and its use does not lead to an increase in the error of the count The counting chamber must have a flat bottom since a concavity in the bottom of the counting chamber vitiates the phase effect

(4) The chamber and a wet piece of cotton are covered by a Petri dish for 10 to 15 minutes to allow settling of the platelets and to prevent drying of the preparation

(5) Platelets are counted in 10 blocks of small squares (as for RBC counts) 5 blocks being counted in each half of the chamber The total number of platelets so counted times 2500 gives the platelet count per cu mm A 43X phase objective with a long working distance condenser and 10X eyepieces is used In this procedure the platelets stand out as individual round or oval bodies with pink or purple sheen † The presence of any platelet

* The most satisfactory ones are supplied by A H Thomas Co catalog # 0?1 c

† With more fully corrected objectives of recent manufacture platelets may appear a uniform black

3 Preparation of Suspensions of Intact Platelets

E P CRONKITE, G BRECHER and J FURTH

Suspensions of platelets prepared by the method of Dillard et al[1] are 'intact' in the sense that 50-90 per cent of such platelets will circulate for some hours and up to 50 per cent for 24 hours in thrombocytopenic recipients These platelets however, are not necessarily undamaged The observations of Lawrence and Valentine[2] indicate that the survival of cross-transfused cat platelets in a thrombocytopenic cat is 4 days Odell et al,[3] using tagged platelets, found the survival of platelets in the circulation of normal rats to be 5 days Under similar experimental conditions the platelets from suspensions prepared by Dillard's method did not circulate longer than 48 hours as a rule Platelets in the circulation are probably flat discs which appear lancet shaped only when seen on edge and are presumably free of 'pseudopodia' or 'spicules' [4] Platelets in sequestrene when observed under the phase microscope are occasionally seen to be flat discs However as a rule varying numbers are elongated and have pseudopodia The number of discs decreases rapidly and after a few hours all have pseudopodia

Apparatus Required Refrigerated centrifuge set at 3-5° C Siliconed receptacles for whole blood and platelet suspension sizes depending on volumes to be processed Fifteen or 17 gauge electropolished Fenwal laminar flow needles Nonwettable plastic tubing Siliconed pipets

Transfer sets consisting of a suitable length of siliconed glass tubing attached to a plastic tubing and a 5 ml syringe The free end of the glass tube is sealed and provided with a lateral opening about 1 mm in diameter as close as possible to the sealed end of the tube All glassware made pyrogen free by exposure to 170 C for 2 hours Plastic tubing made pyrogen free by washing with super-oxydol and thorough rinsing in pyrogen free water

Solution Required 1 to 1 5 per cent Na_4 sequestrene (Ethylene-diamine-tetraacetate EDTA) in 0 7 per cent sodium chloride in pyrogen free water, adjusted to pH 6 5 by addition of NaOH

Procedure (1) Bleed dogs under pentothal anesthesia from unexposed femoral artery through Fenwal needles and plastic tubing into chilled siliconed bottle containing 10 ml of sequestrene solution for each 100 ml of blood In man, phlebotomy with similar needle and tubing gravity flow are employed In small laboratory animals a satisfactory suspension may be obtained after suitable anesthesia by cardiac puncture, with or

The reproducibility of the counting procedure should be checked from time to time using statistical tests[1 2 4]

There is, at present, no satisfactory method of preserving whole blood by addition of *any* anticoagulant for *later* platelet counts Only counts done on samples which are diluted in the pipets at the time of collection and kept in a rotor for not more than 8 hours are known to be unbiased

It has been suggested that shaking devices other than rotors may introduce unexpected errors Opinions on this point are divided and no adequate data are available to decide at present whether or not other shaking devices may be safely substituted for the Bryan Garrey pipet rotors

Blood from a finger puncture gives counts with only negligible downward bias (average 2 5 per cent lower), but with twice the error of the venous platelet count (If using blood from finger puncture dilute immediately in RBC pipet, following steps 2 to 5 above)

It is conceivable that unusually 'sticky' platelets may clump before the blood can be diluted even when all steps outlined above are adhered to Difficulties in obtaining satisfactory specimens for counting and preparing platelet suspensions from dogs have been observed in the recovery phase of radiation induced thrombocytopenia in dogs There are, at present, no well documented reports of such occurrences in man

In general the errors other than the minimum statistical errors, in platelet counts are due to one of the following (a) careless venepuncture and collection (b) delay in dilution (c) unconscious equalization of results when counting and (d) selection of areas to be counted when using indirect methods

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ously Whether glucose improves platelet survival has not been tested Only 50-90 per cent of the platelets prepared by Dillard s method are "viable' and are not necessarily equivalent to platelets in the circulation or even platelets in whole blood collected in EDTA

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4 Separation of Platelets from Blood

L M TOCANTINS

The anticoagulants most commonly employed for this purpose are citrates and the oxalates To keep the platelets intact the collection of blood should be as rapid as possible and any contact with wettable surface avoided If it is desired to dilute the blood as little as possible concentrated solutions of anticoagulant (20 per cent sodium citrate or 25 per cent potassium oxalate) may be used but unless the blood is collected rapidly and mixed with the anticoagulant at once many platelets are altered in these solutions

In Man Place the amount of anticoagulant solution necessary to keep the blood from clotting in the syringe and wet the sides of the syringe thoroughly with it If the solution is sterile wet the interior of the needle as well It is advisable to use an excess of an isotonic solution of the anticoagulant and a syringe of 50 ml capacity With a sharp needle of short shank and wide bore puncture the vein and draw the blood swiftly into the solution Collect approximately 30 ml empty the contents into two paraffin coated rounded bottom centrifuge tubes in each of which there has been placed approximately 25 ml of a mixture of citrate (or oxalate) and physiologic solution of sodium chloride Mix thoroughly by pouring from one paraffin-coated tube into the other Centrifuge at about 1 000 revolutions

without exposure of the heart, with needles of suitable size, and collection of blood by very gentle suction into siliconed syringe containing prescribed amount of sequestrene solution Intravenous injection of heparin before bleeding or exsanguination has proved useful in rats and increases the platelet yield The amount of sequestrene may be reduced under these circumstances

(2) *Centrifuge at 30 g for 50 minutes to sediment RBC* (400 to 500 rpm in an International centrifuge with 4 Dural cups for 200 ml bottles)

(3) *Transfer plasma into second siliconed centrifuge bottle or tube* For this purpose, the glass tube of the 'transfer set' is kept near the bottom of the plasma layer, 2 to 3 mm above the RBC layer The syringe is held below the level of the plasma layer, and plasma aspirated until it nearly reaches the tip of the syringe The plastic tubing is momentarily clamped the syringe removed and the plasma allowed, by syphon effect, to flow into the clean siliconed centrifuge tube or bottle

(4) *Centrifuge plasma at 300 g for 45–60 minutes* (1500 rpm in an International centrifuge with 4 Dural cups for 200 ml bottles)

(5) *Decant rapidly all but a small amount of plasma*

(6) *Resuspend packed platelets in small amount of plasma by repeated aspiration and expulsion of plasma in siliconed pipet* Rather vigorous loosening of packed platelets from the bottom of the centrifuge bottle by "scraping the bottom" with tip of pipet may be required

Precautions and Sources of Error The method must be suitably modified to insure sterility if it is to be used in man * Platelet groups very likely exist in man and possibly in other mammals and immunization against homologous platelets is likely to occur with suspensions prepared from cross-matched blood The method has not been subjected to a critical evaluation of the importance of refrigeration to 3–5° C nor the importance of pyrogen free technic

The speeds and times of centrifugation were evaluated and are those found satisfactory in preparation of suspension of dog platelets for transfusion into thrombocytopenic dogs There are individual variations in the rate at which platelets are sedimented by centrifugation in bloods from different animals or from different bleedings In some cases recentrifugation may improve the yield Probably, isotonic Na_2 sequestrene (5 per cent in distilled water 3 ml per 100 ml of blood) could be substituted for the solution used by Dillard et al [1] The addition of glucose in concentration similar to that in ACD solution does not interfere with the yield and makes it possible to collect and separate platelets and red blood cells simultane

* This has recently been accomplished by Gardner Howell and Hirsch [8] using a closed system of special plastic bags and transfer sets made by Fenwal Laboratories Framingham Mass

vein in the unanesthetized animal or from the carotid artery From a dog weighing approximately 15 kilograms 200 cc of blood may be collected from the heart without any disturbing symptoms The puncture is made with the animal s head immobilized and the body firmly stretched and bound on a board The syringes are loaded with the solution and laid by the side of the operator Since the puncture is made almost vertically a brake is placed on the piston to keep the solution from flowing out The needle is left in the heart while syringes are being shifted

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5 Volumetric Measurement of Platelets

L M TOCANTINS

Only an approximate idea of the total volume of platelets in the blood may be obtained from determinations of the percental volume as currently done Such determinations may be expected to give an indication only of the volume of platelets in the blood in the vessel from which the blood was collected for the number of platelets varies in different vessels and regions of the body The technic of Van Allen may be modified so as to give the average volume of each platelet and knowing the number of platelets per cubic millimeter in a particular sample of blood one may calculate the per cental volume in this sample In general the objections to Van Allen s method (which seems the best available) are principally that some leuko cytes may be packed down with the platelets thus increasing the volume of the platelets and since (if Van Allen s solution is used) the platelets are not fixed their volume may change on withdrawal of the blood and standing of the specimen for any length of time

MODIFIED VAN ALLEN METHOD

Apparatus A thrombocytocrit * a 10 ml record syringe and a centri fuge capable of 3 500 revolutions per minute

Draw 6 ml of a filtered solution containing sodium citrate 3 8 per cent and solution of formaldehyde U S P 0 1 per cent into a record syringe With a sterile needle gauge between 20 and 22 withdraw 4 ml of blood rapidly without stasis into the solution Remove the needle and tilt the

* The Van Allen thrombocytocrit may be obtained from the A H Thomas Com pany Philadelphia Pennsylvania

per minute for from four to five minutes Pipet off the supernatant fluid, which is of a light pink color, into paraffin coated conical bottom centrifuge tubes and centrifuge for an hour at about 2 000 revolutions per minute Pipet off the supernatant fluid and discard it The accumulated sediment appears distributed in two layers a thin layer of red blood cells and superimposed on it a thick white layer of platelets among which are mixed a few white and red blood cells Cover the sediment gently with approximately 10 ml of cold physiologic solution of sodium chloride By applying suction through a glass pipet (capillary tubing drawn out to a tip measuring about 1 mm in internal diameter), remove the white layer carefully, beginning with the central portion and gradually working toward the periphery and more deeply toward the packed layer of red blood cells Place the mixture of salt solution and platelets into a 15 ml conical bottom glass tube and emulsify gently by aspirating and expelling it with a dropping rubber bulb pipet Avoid bubbling After the solution is free from coarse floating particles and has an even pale blue translucent appearance centrifuge it again for one hour at 2,000 revolutions per minute or until the supernatant fluid is clear After the sediment is again packed remove the supernant fluid replace it with cold physiologic solution of sodium chloride and pipet off the white layer of platelets into another 15 ml centrifuge tube repeat the gentle emulsification and centrifugation At least three centrifugations of the emulsified sediment are usually necessary to rid it of most of the white and red blood cells Frequent microscopic observations of the emulsion should be made to make sure that the white and red blood cells are being removed In pipetting off the white layer, no attempt should be made to remove it entirely down to the level of the red blood cells if a white film is left over the packed red cells most of the white cells will have been eliminated By this process approximately two thirds of the collected platelets are isolated

The amount of blood collected may be adjusted depending on the purpose for which the platelets will be used Greater amounts may be isolated by having several syringes loaded with the anticoagulant solution and switching from one to another the needle being left in place until the desired amount of blood is collected

All glassware must be scrupulously clean the solutions filtered and cold *All contact with cotton or thready material must be avoided* such material should not be used as stoppers for the bottles containing the solution The collection of blood should be made early in the morning to allow sufficient time for the various steps during the rest of the day The platelet emulsion while not in use should be kept in the ice chest at a temperature of approximately 5° C

In Animals Platelets may be isolated from the blood of animals in a similar manner, the blood being collected by puncture of the heart or jugular

The percental volume of the platelets in whole blood (thrombocytocrit) is calculated from the mean platelet volume (12 cubic microns) multiplied by the number of platelets per cubic millimeter of blood (first platelet count) multiplied by 100

$0.000\,000\,012 \times 300\,000 \times 100 = 0.36$

Percental volume of platelets = 0.36 per cent

Values Found average volume of platelets in the blood of man 0 49 per cent (range 0 35-0 67)

Reference

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6 Estimation of the Clot-Accelerating Action of Platelets

J H MILSTONE

Object To attempt to determine how platelets are related to the production of thrombin

Principle Blood is drawn with care to limit platelet changes The platelets are then separated from the other formed elements washed twice to remove plasma and tested in systems containing purified components

Reagents apparatus and preparation of platelet suspensions

(a) Buffered Saline pH 7 4 (Buffer)

NaCl	9 Gm
0 1M Sod diethylbarbiturate	200 ml
0 1M HCl	144 ml
Boiled distilled water	to 1000 ml

(b) Platelet Suspension Twenty five ml rabbit blood is drawn from the heart into a syringe containing 25 ml cold acid-citrate (37 5 g 2 $Na_3C_6H_5O_7$ 11 H_2O plus 1 05 g $H_3C_6H_5O_7$ H_2O per liter of solution pH 6 3) The platelets are separated by 3 cycles of differential centrifugation during which they are washed twice with cold buffered saline pH 7 4 The centrifugations are carried out in $9\frac{1}{2} \times 1\frac{1}{2}$ cm lusteroid tubes The 3 slow centrifugations are at 1100 rpm in an International Size 1 Type C centrifuge at room temperature whereas the 3 alternate fast runs are made at

syringe two or three times to insure good mixing Place the diluted blood in a paraffin-coated centrifuge tube of 15 ml capacity and mix the contents gently for five minutes in an automatic shaker Perform a platelet count on this mixture, making allowance in the calculation for the original dilution used Centrifuge at 1 000 revolutions per minute for five minutes if the blood is of normal erythrocyte content, with blood containing from one half to one third the normal number of erythrocytes, centrifugation for from two to three minutes is sufficient for separating the red and white cells With a paraffin-coated pipet remove approximately 2 5 ml of the platelet rich fluid to another paraffin coated tube This is to be corked and shaken gently for five minutes in an automatic shaker The second platelet count is done on this suspension Draw exactly 2 ml of the suspension into the thrombocytocrit by applying a mouth suction tube over its upper end and dipping the graduated end into the measured solution Before beginning the aspiration, place the ring of the metal clip over the upper end of the thrombocytocrit and allow the metal bucket with its rubber base to hang loose Immediately after all the fluid has been aspirated, apply the metal bucket to the bottom of the graduated tube Then place the thrombocyto crit in a centrifuge cup and balance it with another thrombocytocrit filled with 2 ml of water as a counterweight Centrifuge at 3,500 revolutions per minute for one and a half hours, at which time the capillary lumen of the thrombocytocrit is found partly filled with a white sediment, the super natant fluid being clear Read the actual amount of white sediment directly from the scale on the tube in cubic millimeters Divide this figure by the *total number* of platelets in the centrifuged plasma The result gives the mean volume for the individual platelet To obtain the volume of the platelets per hundred cubic millimeters of blood (percental or thrombocytocrit volume) of the sample collected it is necessary only to multiply the volume of the individual platelet by the number of platelets per hun dred cubic millimeters of blood

For example

Number of platelets per cubic millimeter of blood 300 000

Volume of packed platelets in cubic millimeters 13

Platelet count per cubic millimeter of centrifuged plasma 530 000

Amount of centrifuged plasma 2 ml

Total number of platelets in the centrifuged plasma

$2 \times 530\,000{,}000 = 1\,060\,000\,000$ platelets

$$\text{Mean platelet volume} = \frac{13}{1\,060\,000{,}000} = 0\,000\,000\,012 \text{ cubic milli}$$

meter = 12 cubic microns

may be obtained with one of the numerous modifications of the old two-stage test as used for studying the rate of thrombin production (cf Arthus 1901 and Mellanby 1917) In the first stage the various materials are mixed with buffer to give a final volume of 1 1 ml or in some experiments 1 0 ml At intervals a 0 1 ml sample is mixed with 0 3 ml oxalated fibrinogen and the clotting time recorded To convert to relative thrombin concentration the arbitrary number 3 000 was divided by the clotting time These relative values were not intended to have a fixed relation to an arbitrary unit of thrombin The general principles to be outlined can be demonstrated with other modifications of the two-stage technic the important consideration is the purity of the materials

Results (a) In the presence of calcium washed rabbit platelets plus a small amount of bovine globulin activate bovine prothrombin [4] As evident from figure 1 the effect of platelets plus globulin is much greater than the

Activation mixtures

0 3 ml	0 4 ml	0 1 ml	0 1 ml	0 1 ml
Buffer	Prothrombin	Buffer Platelets Platelets Platelets	$CaCl_2$ 0 0075M	Globulin 1/30 Buffer Globulin 1/30 Globulin 1/100

0 1 ml of each mixture added at intervals to 0 3 ml oxalated fibrinogen

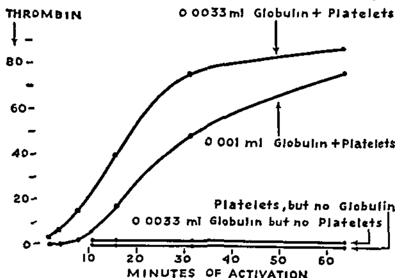


FIG 1 —Activation of prothrombin by globulin plus platelets

3400 rpm in a Sorvall, Type SP angle centrifuge kept in a refrigerator at 2–4° C After the first slow 20 minute run the turbid plasma is pipetted off and the red cells are discarded A 30 minute fast run serves to sediment the platelets out of the plasma, along with some red and white cells The sediment is suspended in 20 ml cold buffered saline with the aid of a glass pipette and the suspension is centrifuged slowly for 10 minutes to remove most of the remaining red cells, along with some platelets The turbid supernatant is subjected to a 20 minute fast centrifugation, and the resulting sediment is resuspended in 20 ml cold buffered saline The ensuing fifth and sixth runs are like the third and fourth The final platelet sediment is suspended in 2 5 ml buffered saline Microscopic examination should reveal no white cells and only one red cell per several hundred platelets Wright stained preparations should show platelets with the characteristic reddish purple granules, but the blue hyalomere is observed only occasionally

(c) Purified Prothrombin The tests require a prothrombin preparation that is not readily activated by platelets plus calcium ions Prothrombin prepared by adsorption on either $Mg(OH)_2$ or $BaSO_4$ has been satisfactory provided that other steps were included in the procedure (see page 112)

(d) Frozen Euglobulin Frozen euglobulin may be obtained from Armour and Company of Chicago Citrated bovine plasma is diluted with 10 volumes of cold tap water and the pH brought to 5 1 by addition of 1 per cent acetic acid After settling overnight in the cold the supernatant is discarded and the precipitate separated and stored at −17° C The weight of the frozen precipitate from 100 liters of plasma is usually between 4 and 6 kg

(e) Globulin Six Gm frozen euglobulin is triturated with 40 ml buffered saline 2 5 ml NaOH N/10 is added to bring the pH to 7 4 by phenol red The mixture is centrifuged to remove undissolved material and the supernatant is readjusted to pH 7 4 by adding 0 1 ml NaOH N/10 38 ml supernatant is mixed with 3 8 ml $CaCl_2$ 0 0275M and the mixture is poured into four conical centrifuge tubes of 12 ml capacity, where coagulation should occur in five minutes The clotted mixture is stored in the refrigerator Later the same day, the mixture is brought to room temperature and the clots are broken up with a stirring rod The mixture is centrifuged yielding a clear supernatant which is used as globulin The globulin' contains appreciable thrombin

(f) Thrombokinase Prepared from frozen euglobulin essentially as described on page 63

(g) Trypsin 150 mg of crystallized trypsin (Armour) containing about 60 mg protein and 90 mg $MgSO_4$, is dissolved in 8 ml HCl 0 001M dialyzed against HCl 0 001M in the cold and diluted to 10 ml

Steps in performance of the tests The particular results to be described

TABLE 2 *Activation of Prothrombin by Trypsin with or without Added Platelets*

Activation mixtures					Period of activation			
0 4 ml.	0 4 ml.	0 1 ml.	0 1 ml	0 1 ml.	4 min.	8 min	16 min.	32 min.
Buffer	Prothrombin	Platelets	$CaCl_2$ 0 0275M	Trypsin 1/6 000	1200	300	10[illegible]	60
		Platelets		Buffer	—	—	—	—
		Buffer		Trypsin 1/6 000	—	—	—	>900
		Buffer		Trypsin 1/600	415	200	115	78
		Buffer		Trypsin 1/60	67	80	165	>900

Figures in body of table give clotting time in seconds of 0 1 ml sample mixed with 0 3 ml oxalated fibrinogen The dash lines indicate that the mixture did not clot in ½ hour The stock trypsin containing 6 mg protein/ml was diluted in buffer shortly before the experiment The last test illustrates how rapidly thrombin activity fell in the presence of 10 micrograms trypsin/1 1 ml

lets are refractile and smooth contoured When a good suspension of platelets activates a prothrombin reagent in the presence of calcium the prothrombin is suspected of being contaminated with thrombokinase or closely related material Thrombokinase may be prepared routinely as a by product of prothrombin purification

The experiments must be arranged or controlled so that a magnification of thrombin activity is not mistaken for an accelerated production of thrombin In addition to the platelet material which accelerates production of thrombin Ware Fahey and Seegers[10] have reported that another platelet factor decreases the time required for thrombin to clot fibrinogen Their test system contained biologicals of bovine origin only Also in the case of calcium it is necessary to distinguish the effect on apparent thrombin activity from the effect on rate of thrombin production [6]

Beyond this the observed rate of thrombin production is a net rate depending on the thrombin produced and on the thrombin concurrently lost by inactivation or by other effects

It is quite possible that platelets have two or more factors which accelerate production of thrombin [9]

Whether some factor which really belongs to the platelets is easily lost by washing is a question to be considered after the more obvious facts have been delineated

Useful references Travis and Ferguson[8] have published findings somewhat similar to those described here and they have extended the technique by adding serum Ac globulin to the test system Alexander[1] has de

sum of their separate effects Neither the platelets nor the globulin cause noticeable activation of prothrombin Occasionally platelets calcium and the prothrombin produce thrombin very slowly during more prolonged incubation, but this may be due to a small amount of globulin factor contaminating the platelets or the prothrombin In the quantity used no activation may be detected with the globulin alone Larger quantities of globulin cannot be used because the contaminating thrombin would interfere too much

(b) Thrombokinase partially purified from the same type of frozen euglobulin as that used to prepare the above globulin reagent activates prothrombin without the addition of calcium ions or platelets When calcium and platelets are included *production of thrombin is much faster* When the calcium chloride is omitted the addition of platelets to the prothrombin thrombokinase mixture makes comparatively little difference as seen in table 1

(c) In the presence of calcium, washed rabbit platelets plus a small amount of trypsin activate bovine prothrombin [8] Larger amounts of trypsin activate prothrombin without the addition of calcium ions or platelets However as illustrated by table 2, the effect of platelets plus a small amount of trypsin is much greater than the summation of their separate effects

Summary and Conclusions In the presence of calcium washed platelets cause little or no activation of suitably purified prothrombin In the production of thrombin from prothrombin platelets do not function in the same way as thrombokinase or as trypsin Rather (a) Platelets complement the effect of certain globulin preparations (b) Platelets complement the effect of thrombokinase preparations (c) Platelets complement the effect of crystallized trypsin (d) Platelets require the addition of calcium in order to be effective as a complement to thrombokinase preparations

Precautions and Sources of Error Best results are obtained when the heart puncture is successful at the first thrust and when the washed plate

TABLE 1 *Activation of Prothrombin by Thrombokinase with or without Added Calcium and with or without Added Platelets*

Activation mixtures					10 min	20 min	40 min
0.3 ml.	0 4 ml.	0 1 ml.	0 1 ml	0 2 ml.			
Buffer	Prothrombin	Buffer	$CaCl_2$ 0 0275M	Thrombokinase 1/16	360	215	155
"		*Platelets*	*$CaCl_2$ 0 0275M*		*45*	*33*	*32*
		Buffer	Buffer		560	410	245
		Platelets	Buffer		540	355	215

Figures in body of table give clotting time in seconds of 0 1 ml sample mixed with 0 3 ml oxalated fibrinogen

7 Estimation of the Adhesiveness of Blood Platelets (Method of Wright)

Adapted by R R HOLBURN*

Object of the Method The measurement of the degree to which platelets adhere to certain surfaces

Principle Blood platelets in vitro progressively decrease in number as time following withdrawal increases Platelets adhere readily to glass surfaces and the rate of disappearance from blood samples in contact with glass serves as an index of their adhesiveness

Apparatus and Reagents Waxed beakers syringes thinly coated with liquid paraffin special glass tubes with a central bulb for rotating blood samples (see original article) Rees Ecker diluting fluid containing 2 per cent formalin instead of 0 2 per cent Bürcher Counting Chamber red cell pipets

Procedure 5 ml blood is drawn into a syringe thinly coated with paraffin and immediately transferred into a waxed beaker containing 0 2 mg heparin per ml blood The beaker is gently agitated to mix the contents Within 5 minutes 2 ml of the heparinized blood is transferred to a *special glass tube* which is mechanically rotated for 80 minutes Platelet counts are made on the blood initially and on samples drawn from the rotating tube every twenty minutes The platelet counts are done by the direct wet method the whole field of the Burcher chamber being counted In order to prevent adhesion of the platelets to the dry stem of the pipet the diluting solution is first drawn up to the 0 5 mark and the blood then drawn into this up to the 1 0 mark A control sample from a known normal individual is treated the same way

Calculation The number of platelets expressed as percentage of the initial count is plotted against the time of rotation in 20 minute intervals The estimation of platelet stickiness of the unknown blood is estimated by comparison with the control sample under identical conditions

Values Obtained Normal average 36 per cent of normal platelets remain free (range 31–42 per cent) after 80 minutes of rotation of the tube

Precautions and Sources of Error (1) Since platelets adhere readily to glass surfaces the use of wax or vaseline surfaces is required in the initial steps of the procedure In collecting the platelet counts for the same reason it is important that the diluting fluid be drawn into the pipette first then the blood drawn into it

(2) Since the percentage of platelets removed is dependent upon the

* From Lancet 1 306 1946

scribed the combined effect of platelets plus an Spca preparation and he has reported his quantities of platelets and Spca in terms of micrograms N/ml The interpretations preferred by the various authors appear in the references quoted The review of Tocantins[7] gives a fuller picture of the breadth and scope of the platelet problem

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to each side of the animal (a) and (b) decrease the dose rate For practical purposes dose rates less than 6 r per minute are undesirable because the effect at lower rates becomes dependent upon dose rate In the event that *the dose rate becomes too low the only alternative is to use higher energy* radiation

For mice rats guinea pigs and to a limited extent rabbits the stand ard 200 250 and 400 kvp therapy x ray machines are satisfactory when some or all of the modifications above are utilized For larger animals it is difficult to get sufficiently large fields with satisfactory depth dose and adequate dose rates

In practice the dose is split equally to both sides of all mammals except the mouse For the induction of thrombocytopenia in dogs of all sizes the following procedure has yielded satisfactory reproducible and quantitative results (a) 2 0 mev G E industrial x ray machine* (b) radial beam (c) TSD 2 0 meters, (d) Dose rate 14 r/min (e) HVL 4 3 mm Pb (f) Effective energy approx 500 kev, (g) Turn dogs around after delivering half of dose

The degree of thrombocytopenia and the time of recovery are a function of dose as are mortality and survival time [3] At higher doses the survival time becomes so short that thrombocytopenia does not develop For reference purposes the relationship of dose to mortality survival time and bleeding tendency is shown in table 1

With doses that produce survival time of less than 6 days, thrombocytopenia does not develop Between 150 and 1000 r a definite thrombo cytopenia develops From 500–1000 r the response is similar and maximal For all doses the platelet count may trend upwards for a day or so By the 5th day the count is decreasing and minimum levels are attained by 9–12 days and a relatively constant level is maintained for 2–3 weeks at which time recovery begins or the animals may die at any time from the sequelae of pancytopenia The characteristic response of the dog is tabulated

D se	Tim to M m m (D y)	D t f Ste dy Stat Th ombop	A rag Le l f Ste dy St t	Recov ry B g
150	10–12	10–20 days	100 000/mm^3	3rd–4th wk
-00	10–1[illegible]	10–20	50 000/mm^3	
00	10–1[illegible]	10–20	30 000/mm^3	
400	9–11	10–30	15 000/mm^3	4th–5th
600	9–10	From 10th day to death	0–5,000/mm^3	None

Thrombocytopenia can also be induced by chronic exposure to radia

* 1 0 mev G E x ray machine has worked equally well as has cobalt gamma rays Less powerful machines can be used but reproducibility is less accurate

concentration of anticoagulant present, the procedure should be followed as described *The higher the concentration of anticoagulant, the less adhesive are the platelets*

Discussion The progressive fall in platelet counts of blood in vitro is mainly, if not entirely, due to their adhesion to the glass walls of the container *rather than disintegration The platelets in the cases of hemophilia* studied are significantly less adhesive than the normal platelets

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8 Induction of Thrombocytopenia in Animals by Ionizing Radiation

E P CRONKITE

Fabricius Moller[1] *clearly demonstrated in 1922 that a severe thrombo*cytopenia could be induced by a single dose of ionizing radiation in guinea pigs He further demonstrated that shielding of the legs lessened the severity of the thrombocytopenia The latter study is the key to the successful and *uniform induction of any desired degree of thrombocytopenia in animals* The exposure to radiation must be uniform over the whole body with a relatively constant depth dose so that all hemopoietic tissue absorbs relatively the same amount of energy These basic considerations necessitate *more extensive and frequent calibration of equipment than is usually* performed for therapeutic x ray The field of radiation must be measured carefully in all quadrants to determine the variation in intensity Variation of greater than ±2 per cent should not be permitted With some x ray tubes it is necessary to alter the cones or build parabolic filters in order to get large fields of uniform intensity The next physical consideration of prime importance is a constant depth dose in the animal This is determined by measuring the dose at various depths in material of electron density similar to tissue and of approximately the same size as the animals to be exposed If the fall off in intensity due to absorption and inverse square is greater than 10 per cent it must be compensated for by some or all of the following maneuvers (a) increase distance from anode to skin (minimize inverse square), (b) add filters to increase effective energy (c) split the dose half

CHAPTER VI

PLASMA THROMBOPLASTIN AND PRECURSORS

1 Demonstration of Platelet Cofactor Activity in Plasma (Method of Johnson)

Adapted by R R HOLBURN*

Object of Method Demonstration of the presence of a plasma globulin which in conjunction with a platelet extract activates purified prothrombin to thrombin Measurement of thromboplastin like activity arising from the interaction of platelet extract and the plasma globulin

Principle If a mixture of platelet extract calcium and purified prothrombin is made and a specially treated plasma is added the amount of thrombin formed will depend on the amount of platelet cofactor available in the plasma The activity formed can substitute for tissue thromboplastin

Apparatus and Reagents Purified prothrombin Prepared according to the method described elsewhere (see page 112) Solution made to contain about 3 000 U/ml

Platelet extract Platelets are obtained by differential centrifugation and washed three times with 0 85 per cent NaCl One part of the packed platelets is mixed with 9 parts of 0 85 per cent NaCl and frozen Then 10 ml of the frozen suspension is thawed and centrifuged (1500 g for 30) in the cold The sediment is recovered washed once in 0 85 per cent NaCl and resuspended in 2 ml of 0 85 per cent NaCl

Plasma samples A clean venepuncture using a siliconed syringe is made and the first syringe replaced with another after 2 ml of blood is drawn and discarded Then 10 ml of blood are placed immediately in a silicone coated centrifuge tube containing 1 0 ml of 0 112 M potassium oxalate The two are mixed and the plasma is removed after centrifugation (1500 g for 30 at 8 C) Plasma is defibrinated by the addition of an equal volume of thrombin containing 20 U/ml To destroy antithrombin the plasma preparation is mixed with an equal volume of ethyl ether and shaken

* From American Journal of Clinical Pathology 23 875 1953

TABLE 1 *Untreated Dog Mortality—2 m e v (Bilateral Technic)*

Dose ( )	No Exposed	No Survived	No Died	% Mortality	Survival Time in Days			Degree of Bleeding
					Min	Mean	Max	
150	18	18	0	0	—	—	—	±
200	11	8	3	27 3	12	16	21	+
300	15	10	5	33 3	15	19 6	23	2+
350	8	4	4	50 0	10	14 9	20	3+
375	3	0	3	100 0	14	19 0	24	3+
400	26	4	22	84 6	6	17 6	40	4+
450	4	0	4	100 0	14	14 5	15	4+
500	11	0	11	100 0	7	10 0	12	4+
600	49	0	49	100 0	4	11 9	21	4+
800	4	0	4	100 0	9	11 8	17	4+
1000	7	0	7	100 0	3	7 0	9	2+
1500	6	0	6	100 0	0 5	3 1	4	0
2000	6	0	6	100 0	3	3 8	4	0
3000	6	0	6	100 0	3	3 0	3	0

tion Some observers believe the platelet level to be a very sensitive index of chronic exposure to radiation Exposure of the dog to 25 r per day over a 14 hour period results in a severe thrombopenia after an accumulated dose of 600 r without as severe a depression in the leukocyte levels

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[2] Cronkite E P and Brecher G Defects in hemostasis produced by whole body irradiation in Blood Clotting and Allied Problems ' Transactions of the Fifth Conference of the Josiah Macy Jr Foundation 1952 p 171

2 Separation of Thrombokinase from Plasma and Demonstration of Its Activity

J H MILSTONE

Principle Crude euglobulin fractions of slaughter house bovine plasma (and also of human plasma as ordinarily collected in the clinical laboratory) will activate their own prothrombin in the presence of calcium This shows that crude euglobulin contains or can give rise to, thrombokinase Thrombokinase is obtainable as a by product of prothrombin purification and in the present case it is accompanied by very little in the way of accessory factors However it is accompanied by thrombin most of which can be removed by repeated precipitation with ammonium sulfate This thrombokinase can then be tested on purified prothrombin, with or without the restoration of accessory factors

Crude euglobulin is extracted with oxalated saline and the extract is subjected to two successive adsorptions the first by hyflo super cel and the second by barium sulfate Two successive elutions from the barium sulfate are performed The first elution with 0 3M phosphate pH6 6 yields most of the prothrombin The second eluate obtained with 0 6M phosphate pH7 9 gives rise to thrombokinase and thrombin From this mixture, thrombokinase is separated by ammonium sulfate fractionation and dialyzed

The present method appears to be the only one used repeatedly over a period of years to yield a separated blood derivative which can activate prothrombin without calcium ions and which contains little thrombin With this as a beginning it is hoped that simpler methods will follow

Reagents and materials

Hyflo Hyflo super cel a grade of diatomaceous silica supplied by Johns-Manville Corporation New York New York

E & D Filter Paper The Eaton Dikeman Co Mt Holly Springs Pennsylvania

$BaSO_4$ U S P suitable for x ray diagnosis supplied by Merck and Co Rahway New Jersey Some stocks of analytical reagent are not satisfactory for the present procedure

Oxalated Salt Solution	NaCl	36 Gm
	$K_2C_2O_4 \cdot H_2O$	20 Gm
	Distilled water to 2 liters	
4 M NaCl	NaCl	1404 Gm
	Distilled water to 6 liters	

for three minutes The ether extraction is repeated three times The material is stored in the deep freeze

Thrombin Activity Determine according to the method described on page 121

Serum Blood is permitted to clot at room temperature, after two hours it is centrifuged at 1500 g for 30

Procedure Into a silicone coated 10 ml test tube are placed 1 ml of the unknown plasma or serum 0 5 ml $CaCl_2$ (0 153 M) in imidazole buffer, 0 5 ml platelet extract 1 ml purified prothrombin Aliquots are removed at timed intervals with a silicone coated pipette The reacting mixture is suitably diluted to have a final concentration, when added to fibrinogen of approximately 1 unit per ml

Calculation The total units of thrombin formed are plotted on graph paper against the time the aliquot is removed in minutes Normal plasma produces a full thrombin yield in 30 minutes after a few minutes latent period

Precautions and Sources of Error The concentration of prothrombin in the mixture must be sufficiently high so that the prothrombin content of the added plasma may be ignored due to the high dilution of the plasma in the test system

The reacting mixture must be adequately diluted to yield a thrombin concentration low enough to be accurately measured (about 1 unit per ml) The plasma must be shaken with the ether for at least 1½ minutes to destroy antithrombin

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Reagents and materials

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$BaSO_4$ U S P suitable for x ray diagnosis supplied by Merck and Co Rahway New Jersey Some stocks of analytical reagent are not satisfactory for the present procedure

Oxalated Salt Solution	NaCl	36 Gm
	$K_2C_2O_4 \cdot H_2O$	20 Gm
	Distilled water to 2 liters	
4 M NaCl	NaCl	1404 Gm
	Distilled water to 6 liters	

1 4 M KCl pH 6 7	KCl	626 Gm
	0 1 M KH_2PO_4	300 ml
	0 1 N NaOH	210 ml
	Distilled water to 6 liters	

The pH values for this and the following solutions are read with a glass electrode

The readings usually do not vary more than 0 1 unit from the values given

Wash pH 6 7	4 M NaCl	125 ml
	0 1 M KH_2PO_4	250 ml
	0 1 N NaOH	120 ml
	Distilled water to 5 liters	
0 3 M phosphate pH 6 6	KH_2PO_4	81 Gm
	1 N NaOH	275 ml
	Distilled water to 2 liters	
0 6 M phosphate pH 7.9	KH_2PO_4	163 Gm
	1 N NaOH	1110 ml
	Distilled water to 2 liters	
SAS Saturated ammonium sulfate		
0 1 SAS 0 1 M acetate pH 5.25	SAS	100 ml
	4 M CH_3COONa	21 ml
	4 M CH_3COOH	4 ml
	Distilled water to 1 liter	
0.35 SAS 0 1 M acetate pH 5.25	SAS	350 ml
	4 M CH_3COONa	21 ml
	4 M CH_3COOH	4 ml
	Distilled water to 1 liter	
0 45 SAS 0 1 M acetate pH 5 25	SAS	450 ml
	4 M CH_3COONa	21 ml
	4 M CH_3COOH	4 ml
	Distilled water to 1 liter	

Preparation of Thrombokinase as a By product of Prothrombin Purification[7]

Preparation of Major Fractions Frozen euglobulin may be obtained from Armour and Co , of Chicago Citrated bovine plasma is diluted with 10 volumes of cold tap water and the pH brought to 5 1 by addition of 1 per cent acetic acid After settling overnight in the cold the supernatant is discarded and the precipitate stored at −17 C The weight of the frozen precipitate from 100 liters of plasma is usually between 4 and 6 kg To avoid continuous changes in procedure 540 gm is arbitrarily taken as roughly equivalent to 10 liters of plasma

Five hundred and forty Gm of precipitate is broken up with a cold chisel passed through a meat grinder and mixed with 900 ml distilled water plus 1 liter of cold oxalated salt solution This mixture is stirred electrically with a glass rod bent at the bottom into a wide triangle thereby

moving the entire suspension without causing much foaming During the stirring about 170 ml 0 1 N NaOH is added dropwise until a phenol red spot test matches that of a pH 7 4 buffer The mixture is centrifuged and the residue set aside To the supernatant is added 400 gm hyflo which is kept suspended by occasional stirring for 20 minutes A suspension of 100 gm hyflo in 500 ml wash is used to precoat an E & D No 615 filter paper on a Buchner funnel 24 cm in diameter Finer filter paper is not satisfactory leading to excessive slowing of filtration

The filters are handled as if they were flat chromatographic columns Fluids are poured on in batches and these are kept reasonably separate by waiting until only a thin layer of preceding fluid remains over the filter cake before the next is poured on

The hyflo-globulin mixture is poured on the prepared filter and filtration with suction is performed slowly until the amber globulin just begins to emerge At this point filtration is stopped and about 400 ml filtrate is discarded Filtration is then completed within 10 minutes Now 500 ml wash is drawn into the filter-cake which is then packed by pressing with a spatula Next 1 000 ml wash is drawn into the cake and about 2450 ml of amber filtrate is removed from the flask and stirred with 800 ml cold 4 M NaCl The salted globulin can be processed at once or stored overnight at 4 C Another 1 000 ml of wash is drawn into the cake the filtrate is discarded and the flask rinsed with distilled water 800 ml 1 4 M KCl is poured on the filter and the suction is adjusted so that 20 minutes are required to draw the KCl into the cake The filtrate is discarded Another 800 ml 1 4 M KCl is poured on the filter and filtration completed slowly The 800 to 1 000 ml of opalescent filtrate is Fraction A About 140 minutes are required for the adsorption filtration washing and elution As routine two batches are run from the frozen globulin to Fraction A in 5 hours

Initially difficulty was met in performing two successive adsorptions with globulin solutions which contained so much fibrinogen Apparently this was largely due to the tendency of the fibrinogen to clot during the process This difficulty may be avoided by salting the globulin after the first adsorption and by selecting the proper stock of $BaSO_4$ for different stocks vary in their suitability for this procedure If the salted globulin had been stored at 4 C it is warmed for 15 minutes in a water bath at 26–30 C Meanwhile a suspension of 10 gm hyflo in 300 ml 1 4 M KCl is used to precoat a No 612 E & D filter paper on a Buchner funnel 24 cm in diameter 150 gm $BaSO_4$ is added to the globulin and stirred continuously for *10 minutes Then 60 gm hyflo is added* and stirred well for 2 to 3 minutes Filtration is begun at moderate speed the mixture being poured with care not to disturb much of the precoat After the first few seconds the suction is turned on full and filtration is completed in 15 to 30 minutes The

$BaSO_4$ globulin suspension is followed by 500 ml 1 4 M KCl and the filter flask is emptied Then 1500 ml wash is passed through and the flask is emptied and rinsed with distilled water The first elution is performed by drawing 900 ml 0 3 M phosphate, pH 6 6, through the cake in 30 to 40 minutes The second eluate is obtained in a similar manner with 900 ml 0 6 M phosphate, pH 7 9 Sometimes twice as much hyflo is needed for rapid filtration

Sometimes the procedure is interrupted just before the first elution, and the washed $BaSO_4$ is stored at −17°C Often, the $BaSO_4$ is stored in the freezer after the first elution, and the second eluates may be prepared from the stored cakes, as needed

As an extension of this major fractionation procedure, the following exploratory fractionations may be done

Kinase and thrombin subfractions The second eluates from 6 $BaSO_4$ cakes are pooled For each liter 474 gm solid ammonium sulfate is added, and stirred well The precipitate is collected on a Buchner filter with 33 gm hyflo as filter aid The moist undisturbed filter cake is kept in the refrigerator 23 hours and then at room temperature for ½ hour For the next ½ hour, 600 ml wash is drawn slowly through the cake which is finally pressed with a spatula To 650 ml of amber filtrate is added 351 ml SAS After 2 hours, the precipitate is removed by centrifugation To 980 ml of supernatant is added 435 ml SAS The precipitate is collected by centrifugation dissolved in 40 ml wash kept at 5 C for 1 week, and mixed with 33 ml SAS to give a kinase precipitate and a thrombin supernatant All fractionation is done at room temperature but the fractions are stored at 5 C at the end of each day

The kinase precipitate is extracted with 20 ml 0.35 SAS 0 1 M acetate, and the residue discarded The extract is mixed with 3 9 ml SAS, and the resulting precipitate extracted with 15 ml 0.35 SAS 0 1 M acetate The extract is recentrifuged the next day to remove any precipitate which forms overnight in the cold, and the extract is then mixed with 2 7 ml SAS The resulting precipitate is extracted with 5 ml 0.35 SAS 0 1 M acetate and the extract is mixed with 0 9 ml SAS The resulting precipitate is dissolved in 15 ml wash and reprecipitated with 12.3 ml SAS The precipitate is dissolved in 20 ml wash and reprecipitated with 16 4 ml SAS This last pair of operations, (20 ml wash then 16 4 ml SAS) is repeated to make a total of 12 such precipitations The final precipitate is dissolved in 10 ml wash and dialyzed against several changes of cold veronal buffered saline over a period of 4 days

The principle involved in this separation has been used repeatedly, with many variations in detail The variations in preparation have not as

yet led to essential qualitative differences in the behavior of the kinase preparations However there have been quantitative differences

Tests with thrombokinase The methods of testing were similar to those described on p 53 The veronal buffered saline was the same The stock solution of thrombokinase used for the experiments of the tables and figures contained 10 4 mg solids/ml in addition to the solids of the buffered saline The stock solution of potassium oxalate was 0 0ɔ7 M and had an ionic strength of 0 171, almost the same as that of the buffered saline (0 174) Prothrombin preparation III[8] was used for the tests The lipoid thromboplastin was that fraction of bovine brain soluble in ether but not in acetone[6] made up in a 10 per cent suspension Cephalin prepared by the method of Howell[9] behaved similarly and was used for the experiment in figure 2

As shown in table 1 thrombokinase activates prothrombin in the presence of 0 01 M oxalate the rate of thrombin production depending on the amount of thrombokinase With a constant amount of kinase the rate of thrombin production is not influenced by varying the oxalate over a four fold range as seen in table 2

As shown in table 3 ionic calcium has comparatively little if any influence on the activation of prothrombin by thrombokinase

In systems containing prothrombin and calcium the addition of both thrombokinase and lipoid thromboplastin causes much faster production of thrombin than twice as much kinase or twice as much lipid As further seen in figure 1 lipid and calcium ions cause no activation of prothrombin during the time limits of the experiment Kinase does activate the prothrombin slowly When the calcium chloride is omitted thrombokinase activates prothrombin as fast without lipid as with lipid[7]

TABLE 1 *Activation of Prothrombin by Varying Amounts of Kinase in the Presence of 0 01 M Oxalate*

Act t mixtures				P od f t t n				
0 7 mL	0 1 mL	0 2 mL	0 1 mL	10 m	20 min	40 ml	80 m	160 min
				c	sec	c.	sec	sec
Buffer	Prothrombin	Oxalate	Kinase undil	61	37	24	18	14
			1/2	111	57	35	23	16
			1/4	224	118	61	37	23
			1/8	480	240	136	66	37
			1/16	1040	535	285	155	71
			1/32	2040	1380	655	340	152
	Buffer		Kinase undil	1500	1380	1500	—	1380

Serial dilutions of thrombokinase in buffer Figures in the body of the table give clotting times of 0 1 ml samples mixed with 0.3 ml oxalated fibrinogen

$BaSO_4$ globulin suspension is followed by 500 ml 1 4 M KCl and the filter flask is emptied Then 1500 ml wash is passed through, and the flask is emptied and rinsed with distilled water The first elution is performed by drawing 900 ml 0.3 M phosphate, pH 6 6, through the cake in 30 to 40 minutes The second eluate is obtained in a similar manner with 900 ml 0 6 M phosphate, pH 7 9 Sometimes twice as much hyflo is needed for rapid filtration

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In systems containing prothrombin and calcium the addition of both thrombokinase and lipoid thromboplastin causes much faster production of thrombin than twice as much kinase or twice as much lipid As further seen in figure 1 lipid and calcium ions cause no activation of prothrombin during the time limits of the experiment Kinase does activate the prothrombin slowly When the calcium chloride is omitted, thrombokinase activates prothrombin as fast without lipid as with lipid [7]

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Act t m t es				P od f act tio				
0 7 ml	*0 1 ml*	*0 2 ml*	*0 1 ml*	*10 min*	*20 min*	*40 min*	*80 min*	*160 min*
					sec	sec		
Buffer	Prothrombin	Oxalate	Kinase undil	61	37	24	18	14
			1/2	111	57	35	23	16
			1/4	224	118	61	37	23
			1/8	480	240	136	66	37
			1/16	1040	535	285	155	71
			1/32	2040	1380	655	340	152
	Buffer		Kinase undil	1500	1380	1500	—	1380

Serial dilutions of thrombokinase in buffer Figures in the body of the table give clotting times of 0 1 ml samples mixed with 0 3 ml oxalated fibrinogen

TABLE 2 *Activation of Prothrombin by a Constant Amount of Kinase in the Presence of Varying Concentrations of Oxalate*

Activation mixture				Period of activation			
Buffer	*Prothrombin*	*Oxalate*	*Kinase*	*10 min*	*20 min*	*40 min*	*80 min*
ml.	ml.	ml.	ml	s c.	s c.	s c.	sec.
0 8	0 1	0 1	0 1	135	84	65	43
0 7		0 2		134	92	61	40
0 5		0 4	'	133	81	50	39

Figures in body of table give clotting times of 0 1 ml samples mixed with 0 3 ml *oxalated fibrinogen*

TABLE 3 *Showing that Calcium Chloride Has Little Effect on the Activation of Prothrombin by the Kinase Reagent*

Activation mixtures				Incubated	Added	Then incubated			
0 7 ml	0 1 ml	0 1 ml	0 1 ml	30 m	0 1 ml	½ min	5 min	10 min	20 m
						sec	sec	sec	sec
Buffer	Prothrombin	Ca	Kinase		Buffer	42	42	38	34
		Buffer			Ca	46	42	37	31

Within the limits of error as much thrombin was produced in 30 minutes without added calcium plus ½ minute with calcium as was produced by the control mixture in 30½ minutes with calcium The subsequent thrombin assays show that the mixtures were not completely activated at the time of the critical tests and hence the critical tests measured the amount of thrombin produced in a definitely known time i e 30½ minutes

In systems containing constant prothrombin and constant calcium the combination of three variables—thrombokinase cephalin and bovine serum—causes faster production of thrombin than twice as much of any combination of two out of the three variables [10] This is illustrated in figure 2

Conclusions Thrombokinase as obtained from the euglobulin fraction of slaughter house plasma, can activate prothrombin in the presence of oxalate provided that the thrombokinase is sufficiently purified and concentrated

Calcium ions plus lipoid thromboplastin complement the effect of *thrombokinase* Ionic calcium is not an effective complement without lipoid thromboplastin and lipoid thromboplastin is not effective without ionic calcium Howell s 'cephalin can be used as lipoid thromboplastin

When thrombokinase is complemented by calcium and cephalin ' a further, different acceleratory effect is exerted by bovine serum Whether this different effect of bovine serum corresponds to one or more of the pro

posed new accelerators is left open Whether bovine serum also contains some thrombokinase is left open

Precautions and Sources of Error The major fractionation procedure has been repeated more than 100 times However it does require care and scrupulously clean apparatus It is not recommended for casual trial

The adsorption procedures are adjusted for use on oxalated euglobulin solutions The hyflo adsorption does not work in quite the same way when applied directly to whole plasma

The relationship illustrated by figure 2 cannot be demonstrated unless the prothrombin preparation has a sufficiently low content of the factors to be added

The experiments must be arranged or controlled so that a magnification of thrombin activity is not mistaken for an accelerated production of thrombin For example in the activation of prothrombin by thrombokinase (table 1 page 54) it seems that calcium chloride makes some difference in the activation However the observed difference is due almost entirely to the effect of calcium on the clotting of the thrombin fibrinogen mixtures

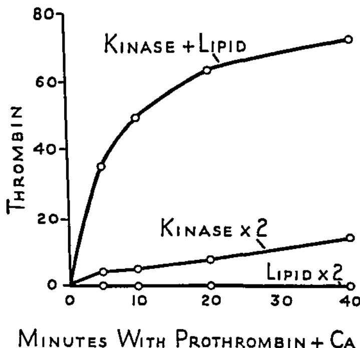


FIG 1 —Test system prothrombin and calcium The activation mixture represented by the top curve had kinase diluted 1/176 and lipid diluted 1/880

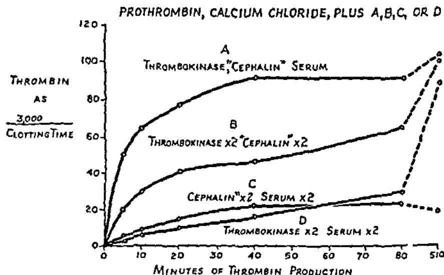


FIG 2 —Test A contained 0 032 mg thrombokinase 0 016 mg Howell s cephalin and 0 00125 ml bovine serum The final volume of all activation mixtures was 1 1 ml The concentration of calcium chloride in the activation mixtures was 0 0025M

When the experiment is arranged to cancel out this difference, shown in table 3, it is seen that calcium exerts little influence on the activation of prothrombin by the particular thrombokinase By an experiment similar to that of table 3, it can be shown that calcium and lipoid thromboplastin together make a real difference in the rate of thrombin production, i e this difference is not cancelled out by adjusting the test and the control to the same concentrations of calcium and lipid just before the samples are assayed for thrombin

Thrombokinase is easily demonstrable in Fraction A, as obtained during the above procedure It can also be prepared in another way [9] But both of these preparations contain at least one accessory factor and hence they are not suitable for these experiments The apparent behavior of thrombokinase has changed as it has been obtained in purer and more concentrated form and certain effects formerly associated with thrombokinase are now seen to be more closely associated with accessory factors It is conceivable that the apparent behavior of thrombokinase will change further as purification progresses

Useful references In his classic review,[11] Morawitz tabulated oxalated plasma as one fluid in which thrombokinase was available In 1909 Mellanby noted, 'The solubility relations of kinase are such as to ensure its precipitation with fibrinogen from bird s plasma ' In 1916 Dale and Walpole prepared thrombokinase by treating stable thrombocyte poor

bird plasma with chloroform Tagnon[12] treated recalcified dog plasma with chloroform, and obtained a derivative that could activate prothrombin in the presence of oxalate There would still be some differences in the interpretation of these observations The history of this topic has been reviewed elsewhere [9]

Alexander's results[1] have suggested that one or more of the proposed new factors can be distinguished from thrombokinase

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3 Estimation of Antihemophilic Activity by the Partial Thromboplastin Time Technic*

R D LANGDELL, R H WAGNER and
K M BRINKHOUS

Object of the Test The diagnosis of hemophilia can no longer be made on the classical triad of family history, prolonged whole blood clotting time and normal bleeding time It has been necessary therefore to develop more specific methods for the diagnosis and study of the clotting defect This test is useful because it is relatively simple to perform yet gives an accurate estimate of antihemophilic activity The test has been of value in both diagnostic and fractionation studies[1 2]

Principle Underlying the Test Certain thromboplastins appear to be unable to compensate completely for the defect of hemophilia These have been termed 'partial thromboplastins' One stage type clotting times done with these thromboplastins have been termed 'partial thromboplastin times The relatively slow clotting of hemophilic plasma with these partial thromboplastins can be accelerated by the addition of small amounts of normal plasma Within limits the shortening of the hemophilic partial thromboplastin time is proportional to the amount of normal plasma present By comparing the relative effectiveness of a known normal plasma and the test material the corrective effect (antihemophilic activity) may be expressed as per cent of normal

Apparatus (1) Syringes and needles (2) Centrifuge tubes (3) Centrifuge (4) Refrigerator (5) Balance (6) Glass tubes 10 x 75 mm are used in determining clotting times Tubes of larger size are necessary for storage of reagents (7) 28 C water bath (8) Serologic pipets A supply of 0 2 1 and 5 ml pipets is needed (9) Stop watch preferably operated with a foot pedal (10) Icebath It is convenient to use a small Dewar flask filled with ice and water (11) Freezer Any standard type freezer cabinet capable of maintaining a temperature of −20 C or lower is useful The freezer is used for storage of thermolabile reagents

Reagents Best results are obtained when the reagents itemized below are used

1 0 11 M sodium citrate
2 0 11 M sodium oxalate

Investigations leading to this method were supported in part by research grants H 1648 and H 1333 from the National Heart Institute Institutes of Health Public Health Service

3 0 11 M calcium chloride

4 0 154 M sodium chloride (normal saline)

5 Imidazole buffer pH 7 2 Weigh out 1 72 Gm C P imidazole (Ed can Laboratories South Norwalk Connecticut) Dissolve in approximately 90 ml 0 1 N HCl Adjust to pH 7.2 dilute to 100 ml Store in freezer

6 Oxalated saline Mix one part 0 11 M sodium oxalate with five parts of normal saline

7 Calcium imidazole solution Mix 7 parts 0 11 M calcium chloride 6 parts imidazole buffer pH 7 2 and 5 parts normal saline Store at −20 C in 10 ml lots

8 Hemophilic plasma substrate Obtain blood from a known hemo philiac using the two syringe method Avoid air bubbles in collection Mix immediately with 0 11 M sodium citrate in a ratio of one part sodium citrate to 8 parts whole blood Centrifuge at about 3 000 g for 20–30 minutes Withdraw the supernatant plasma after determining the hematocrit Keep at 4 C until just before use If the plasma is not to be tested within 1–2 hours after venepuncture store immediately in freezer

9 Oxalated plasma Obtain blood by venepuncture mix immediately with 0 11 M sodium oxalate in a ratio of one part sodium oxalate to 9 parts whole blood The rest of the procedure is the same as in (8) above

10 Barium sulfate treated plasma It is preferable to carry out this procedure at 4 C Add 100 mg Merck Reagent or Baker and Adamson Reagent $BaSO_4$ powder per ml oxalated plasma Adsorb for 30 minutes with occasional mixing Centrifuge at about 3 000 g for 30 minutes With draw the supernatant plasma This plasma should not clot when mixed with thromboplastin and calcium

11 Partial thromboplastin Any of a number of partial thromboplastins may be used in the assay More consistent results are obtained with a crude cephalin preparation which may be prepared from bovine canine or hu man brain as follows

The meninges are removed and the brain is washed under tap water Cover about 100 grams of brain tissue with acetone and let stand for one hour Mash the brain under acetone with a pestle Decant and repeat until the tissue becomes flaky and the acetone remains clear

Grind the tissue with clean sand and spread the resultant paste on a large flat pan Dry for about 15 minutes Cover the dry material with ether and let it stand overnight at room temperature Filter evaporate filtrate to dryness with a stream of air or nitrogen Wash the residue twice with 40 ml acetone and twice with 40 ml boiling acetone Air dry the residue The yield is usually two to three grams

To three grams of the waxy white product add three ml of normal

saline and mix intimately Bring the volume of the suspension to 100 ml by the slow addition of normal saline, with mixing The 3 per cent stock suspension is stored in one ml lots in the freezer A 0 3 per cent stock suspension in normal saline is useful It is also stored in the freezer

Steps in Performance of the Test Test and control plasma are treated with barium sulfate before use

(a) Place hemophilic plasma substrate in the ice bath Bring 0.3 per cent crude cephalin and calcium imidazole solutions to 28°C in the water bath

(b) Prepare 0 015 per cent cephalin suspension by diluting the 0 3 per cent suspension 1 to 20 with normal saline

(c) Using oxalated saline as a diluent prepare 1 per cent 2 5 per cent 5 per cent and 10 per cent dilutions of test and control plasmas One ml of each dilution is adequate When plasma fractions are being tested previous dilutions must be considered in preparing these solutions Variations in plasma dilution caused by varying hematocrits may be corrected for in the final calculations

(d) The dilutions are tested by mixing in order

0 1 ml hemophilic plasma substrate
0 1 ml diluted test or control plasma
0 1 ml 0 015 per cent crude cephalin suspension
0 1 ml calcium imidazole solution

Determine time elapsing from addition of calcium imidazole until clot forms

Clotting times are determined for each dilution of both test and control plasma This should be done in a systematic order rather than at random Consistent results are obtained if determinations are done as follows

1 10 per cent control plasma
2 10 per cent test plasma
3 5 per cent control plasma
4 5 per cent test plasma
5 2 5 per cent control plasma
6 2 5 per cent test plasma
7 1 per cent control plasma
8 1 per cent test plasma

(e) Repeat d twice so that three clotting times are obtained for each dilution of both test and control plasma

Calculations The calculations are illustrated by means of an assay of normal human plasma

(a) Calculate the average clotting time for each plasma dilution

Sample	A Clotting Tim (Sec.)
10 per cent control plasma	130
10 per cent test plasma	141
5 per cent control plasma	153
5 per cent test plasma	165
2 5 per cent control plasma	176
2 5 per cent test plasma	187
1 per cent control plasma	203
1 per cent test plasma	214

(b) Plot clotting time against plasma concentration on semi log paper The plasma concentration is represented on the log scale

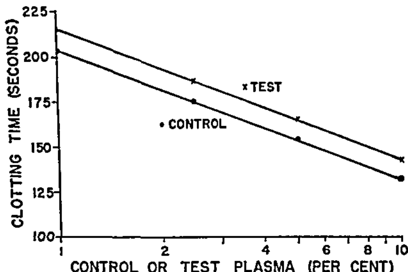


Fig 1 —Graph illustrating m thod of plotting data for calculation of anti hemophilic activity in test sample (From J Lab Clin Med *41* 637 1953)

(c) Determine by interpolation the concentrations of test plasma that would give the same clotting times as 1 2 5 and 5 per cent control plasma

Divide control plasma concentration by the equivalent test plasma concentration and multiply by 100 The average of the results is an expression of the antihemophilic activity of the test sample in terms of per cent of the control

Eq lent Co centratio f Test Plasm (P Ce t)	AHF A t ty as P Cent f Contr l
1 4 per cent	71
3 5 per cent	71
6 9 per cent	72
Average	71

Alternative Procedures (a) Shorter clotting times and sharper end points are obtained by the addition of a very dilute solution of "accelerators" to the 0 015 per cent cephalin suspension The accelerator amounts necessary vary somewhat with the reactivity of the standard reagents but the amounts specified below are usually satisfactory To 5 9 ml of the cephalin suspension is added 0 1 ml of Topical Thrombin 0 5 unit per ml or Russell viper venom, 0 04 μg per ml or serum accelerator, 2 spca units[3] per ml (The modified procedure allows more rapid testing but should not be attempted until one is familiar with the assay)

(b) *When the test sample contains a higher concentration of antihemophilic activity than the control,* it is more convenient to determine the concentrations of test plasma that would give the same clotting times as 2 5 5 and 10 per cent control plasma

Normal Range of Values In a control group of 28 normal adults of both sexes the range was 68 to 165 per cent of the mean and the standard deviation was ±28 per cent

Persons with classic hemophilia have little or no detectable antihemophilic activity i e little or no antihemophilic factor (AHF)

Precautions and Sources of Error (1) Because of the wide range of normal values careful selection of plasma to be used as a control is required Best results are obtained by using the plasma of an individual whose antihemophilic activity is known in relation to the mean of a large group of normal persons or by using pooled plasmas from several healthy adults

(2) Uncontrolled amounts of thrombin or serum accelerator (s) shorten the partial thromboplastin time of hemophilic plasma without regard to the amount of antihemophilic activity It is, therefore essential that the $BaSO_4$ treatment remove these factors quantitatively from the materials to be tested

(3) As the reactivity of the hemophilic plasma substrate may vary, a control must be run with each test sample

(4) The antihemophilic activity of plasma may decrease on standing Therefore after the plasma dilutions are made determinations must be performed promptly

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3 Alexander B Goldstein R and Landwehr G The prothrombin conversion accelerator of serum (SPCA) Its partial purification and its properties compared with serum ac globulin J Clin Invest *29* 881 1950

4 Estimation of Antihemophilic Activity by the Prothrombin Utilization Technic*

J B GRAHAM, G D PENICK and
K. M. BRINKHOUS

Object of the Test To determine the antihemophilic activity of plasma or plasma fractions The results are expressed in relation to the antihemophilic activity of a normal plasma of known AHF (Antihemophilic Factor) concentration

Principle Underlying the Test In classic hemophilia the prothrombin of shed blood is converted to thrombin very slowly [1] After a normal blood transfusion[1] or after addition in vitro of normal plasma to the hemophilic blood [2] the rate at which prothrombin is converted to thrombin is greatly accelerated This corrective action is attributed to the presence in normal blood plasma of a protein antihemophilic factor (AHF) as yet incompletely defined Within limits the amount of prothrombin consumed in the whole hemophilic blood is linearly proportional to the amount of AHF added Thus the amount of AHF required to convert 50 per cent of the prothrombin in a sample of hemophilic blood is the same regardless of the source of the AHF By comparing the amount of test material (AHF) required to cause 50 per cent conversion of prothrombin in the hemophilic blood with the amount of control plasma required to produce the same change the amount of AHF in the unknown can be expressed in per cent of the control AHF of plasma is not adsorbed by $BaSO_4$ [3] while serum accelerator factors which may influence the prothrombin conversion rate in the presence of AHF are adsorbed.[4] For this reason materials tested for AHF are subjected to preliminary $BaSO_4$ adsorption

Reagents and apparatus required (a) Reagents and equipment as outlined for the two-stage determination of prothrombin (page 105) and the prothrombin utilization test (page 117)

(b) An available supply of blood from classic hemophilia either human or canine

(c) Plasmas from control and test subjects Mix 9 parts of blood with one part 0 11 M sodium oxalate solution Centrifuge at 3000–5000 g for 10–15 minutes and obtain the oxalated plasma Mix with powdered $BaSO_4$ in the ratio of 100 mg for each ml. plasma Stir periodically At the end of

Investigations leading to this method were supported in part by research grants H 1648 and H 1333 from the National Heart Institute National Institutes of Health, Public Health Service

Alternative Procedures (a) Shorter clotting times and sharper end points are obtained by the addition of a very dilute solution of 'accelerators' to the 0 015 per cent cephalin suspension The accelerator amounts necessary vary somewhat with the reactivity of the standard reagents but the amounts specified below are usually satisfactory To 5 9 ml of the cephalin suspension is added 0 1 ml of Topical Thrombin, 0 5 unit per ml or Russell viper venom, 0 04 μg per ml, or serum accelerator 2 spca units[3] per ml (The modified procedure allows more rapid testing but should not be attempted until one is familiar with the assay)

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(3) As the reactivity of the hemophilic plasma substrate may vary, a control must be run with each test sample

(4) The antihemophilic activity of plasma may decrease on standing Therefore after the plasma dilutions are made determinations must be performed promptly

References

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whole hemophilic blood the volume is then expressed as ml per ml native hemophilic plasma AHF concentration in the test sample is expressed in terms of per cent of activity of the control This is calculated as follows

$$\frac{\text{ml control plasma per ml native hemophilic plasma}}{\text{ml test sample per ml native hemophilic plasma}} \times 100$$

$$= \%\ \text{AHF in test sample}$$

(b) An example will illustrate the calculations Test plasma was obtained by drawing 9 ml whole blood and mixing with 1 ml 0 11 M sodium oxalate solution The hematocrit was 50 per cent The concentration of the plasma was reduced by dilution with oxalate as follows

$$\frac{\text{volume of native plasma}}{\text{volume of oxalated plasma}} = \frac{4.5}{5\ 5}$$

$$= 0\ 82 \text{ ml native plasma per ml. oxalated plasma}$$

During dialysis the volume of the adsorbed plasma increased from 5 5 ml to 5.8 ml Thus the concentration was reduced further by the factor $\frac{5\ 5}{5\ 8} = 0\ 95$

The hematocrit value of the hemophilic blood was 45 per cent Thus 0 1 ml of each dilution was added to 0 55 ml of native hemophilic plasma The actual volume of each dilution of the AHF-containing plasma added to 1 ml native hemophilic plasma is calculated as follows

Oxalate dilution factor (0 82) × dialysis dilution factor (0 95) × volume of diluted sample added to 1 ml hemophilic blood (0 1 ml) × factor to adjust 1 ml whole hemophilic blood to 1 ml native hemophilic plasma (1/ 55) = 0 142 ml This figure does not take the series of plasma dilutions into account To obtain the actual volume then multiply 0 142 by 1/dilution factor These values are given in the second column in the tabulation below The residual prothrombin in the hemophilic serum mixed with that dilution is indicated in the third column

Plasm Dil tion	ml. plasm /ml nati hemophili plasma	Resid al prothromb per cent.
1 2	0 071	<10
1 4	0 036	<10
1 8	0 018	35
1 16	0 009	58
1 32	0 0045	68
1 64	0 0023	76
Control	0 0000	100

By plotting the figures in columns 2 and 3 on rectangular graph paper [4]

30 minutes centrifuge at about 3000–5000 *g* for 15 minutes Dialyze the adsorbed plasma for two hours with continuous agitation in Visking No-Jax casing against four changes of citrated saline (1 part 0 11 M sodium citrate solution plus 99 parts physiologic saline, i e, 0 154 M NaCl) Centrifugation, adsorption and dialysis are carried out at 4°C

(d) 10 x 75 mm test tubes, graduated at the 1 25 ml mark

Steps in the Assay (a) Collect plasmas as described above Record hematocrit values for each blood sample Also record volume of each plasma before and after dialysis

(b) Place 0 15 ml imidazole buffer pH 7 2 into each of four series of eight graduated test tubes

(c) Prepare serial two fold dilutions of the test and control samples, using saline as diluent For human plasma the series consists of dilutions of 1 2, 1 4, to 1 64 Add 0 1 ml of each dilution of each sample to successive and respective members of the series prepared in (b) This is done in duplicate for each dilution Each tube now contains a 0 25 ml volume of reagents The remaining two tubes in each series are used as controls, 0 1 ml saline is placed into each

(d) Collect hemophilic blood by venipuncture, without anticoagulant, in a silicone treated syringe Immediately place 1 ml whole hemophilic blood in each tube containing the buffer and diluted plasma Sufficient blood should be obtained to fill each tube and to provide a sample from which the hematocrit value can be obtained 0 15 ml 0 11 M sodium citrate is immediately added to the first pair of control tubes, the contents mixed and centrifuged

(e) Invert the other tubes once for mixing and place in a 28 C water bath At the end of 30 minutes add 0 15 ml 0 11 M sodium citrate solution to each tube Gently loosen clot with an applicator stick Centrifuge at once at 3000–5000 *g* for 10 minutes

(f) Remove sera by aspirating with a capillary pipette and attached rubber aspirating bulb Pool the supernatant serum from each pair of duplicate tubes

(g) Determine the prothrombin unitage of each pooled sample by the two-stage method The incubated pair of saline controls is used to verify the conversion defect in the hemophilic blood These should have a residual prothrombin value of 100 ± 10 per cent If the assay is not done immediately freeze (−20°C) samples

Calculations (a) The object of the calculations is to express the amount of AHF in the test plasma in terms of the control First obtain the volume of undiluted test and control samples which when added to 1 0 ml volume of whole hemophilic blood gave a prothrombin half life of 30 minutes (50 per cent prothrombin converted in 30′) Using the hematocrit value of the

5 Estimation of the Clot-Promoting Power of Plasma Euglobulin Prepared by the Method of Progressive Dilutions

L M TOCANTINS, R R HOLBURN and R T CARROLL

Object of the Method A test for the presence and function of clot promoting euglobulins in normal and abnormal plasmas

Principle If the clot promoting euglobulin is present in plasma in normal amount and function it will gain in activity as it is prepared from increasingly more dilute plasma

Apparatus and Reagents *Platelet poor plasma* Use of silicone surfaces throughout the processing and collection is necessary Blood from a clean puncture of a turgid vein using a # 18 needle is drawn into a syringe containing 0 2 ml 19 per cent sodium citrate per 10 ml blood and the mixture well mixed by inversion The citrated blood is allowed to run down the wall of a silicone coated tube by applying gentle pressure to the plunger of the syringe The plasma is separated from the cells by centrifuging at 2400 g for 50 minutes at 4 C and removed by a silicone dropper pipette avoiding the agitation of the cellular elements

Euglobulin solutions Four samples of the platelet poor plasma from the normal and the unknown blood are each diluted with distilled water 1 10 1 100 1 1000 and 1 10 000 Euglobulins are precipitated by the dropwise addition of 1 per cent acetic acid to pH 6 0 After each solution is allowed to stand in the refrigerator for 1 hour it is centrifuged for 30 minutes at 2400 g at 4 C The supernatant is discarded and the sediment redissolved in a volume of 0 85 per cent NaCl solution equal to the original volume of plasma used Alternately the sediments are dried in the dessicator overnight weighed and 0 4 per cent solutions in 0 85 per cent NaCl prepared for testing The pH is adjusted to 7 4 with a drop of imidazole buffer The solutions are tested immediately and should stand in ice before the test period since they will clot if allowed to stand too long

Steps in Procedure The testing is carried out in a 37 C water bath and in silicone tubes Only the pipettes used for plasma need however to be siliconized To 0 5 ml of stable platelet poor plasma 0 1 ml euglobulin suspension 0 05 ml 0 2 M $CaCl_2$ are added and the stopwatch is started at the addition of the calcium chloride Each euglobulin suspension is tested on a substrate of its own plasma as well as normal plasma

Calculation The data obtained is plotted on log log graph paper with clotting times as the ordinate and the plasma concentration from which the euglobulin was prepared as the abscissa values If the euglobulin of the unknown plasma is normal in amount and function the suspensions prepared

and interpolating the ml of the test plasma sample which will give a 50 per cent residual prothrombin level (i e , a prothrombin half life of 30 minutes) is determined This value is 0 0115 ml

From similar calculation with a control plasma it was found that 0 0092 ml per ml native hemophilic plasma gave a prothrombin half life of 30 minutes The AHF in the test plasma was thus

$$\frac{0\ 0092\ \text{ml}}{0\ 0115\ \text{ml}} \times 100 = 80 \text{ per cent AHF}$$

Range of Normal Values For man 50–200 per cent of the mean

Precautions and Sources of Error

(a) Care should be taken to avoid air bubbles and rough manipulations during the collection of the blood inversion of the tubes and loosening of the clots

(b) Prothrombin and proconvertin should be removed completely in the $BaSO_4$ adsorption step A rapid check can be made by doing a one stage prothrombin time determination If clotting occurs in less than 10 minutes readsorb

(c) An occasional hemophilic patient will be refractory to the corrective action of AHF It is believed such patients have developed excessive inhibitors Their blood is not suitable for this assay procedure Similarly, blood from a hemophilic subject who has received a transfusion within the preceding two weeks or a case of mild hemophilia with a normal prothrombin conversion rate should not be employed

(d) *In work with human plasma the subject or subjects used as controls* should be carefully selected We test a group of 15–20 normal subjects and select one or more individuals with values approximating the mean as our standard reference

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Calculation The data obtained is plotted on log log graph paper with clotting times as the ordinate and the plasma concentration from which the euglobulin was prepared as the abscissa values If the euglobulin of the unknown plasma is normal in amount and function the suspensions prepared

and interpolating, the ml of the test plasma sample which will give a 50 per cent *residual prothrombin level (i e, a prothrombin half life of 30 minutes)* is determined This value is 0 0115 ml

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vitro and in vivo to an essentially similar degree as when the euglobulin suspension originates from normal plasma at 10 per cent concentration

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6 Estimation of Plasma Thromboplastin Component (PTC) Activity (Method of White, Aggeler and Glendening)

Adapted by R R HOLBURN*

Object of the Method To express the PTC activity of various plasma and serum fractions in terms of percentage enhancement of prothrombin utilization of blood deficient in this activity

Principle Plasma thromboplastin component is concerned with the production of thromboplastin in the plasma Since this component is associated with the globulin fraction of plasma and serum, a fairly potent concentrate of the factor may be prepared from normal serum by the method of barium sulfate adsorption of acid inactivated serum This fraction when added to PTC deficient plasma restores the defective prothrombin utilization to normal

Apparatus and Reagents Oxalated plasma One volume of 0 1 M potassium oxalate per 9 volumes of blood

Platelet poor plasma Blood is collected as for venous whole blood coagulation time except that syringes are coated with silicone oil The blood is centrifuged at 5000 rpm for 30 minutes in lusteroid tubes in a small angle centrifuge at 4 C The supernatant plasma is placed in lusteroid tubes and centrifuged in a refrigerated ultracentrifuge at 30 000 rpm (75 000 g) for 30 minutes The upper half of the plasma is carefully pipetted off and used

* From Blood 8 101 1953

from the more dilute plasmas will gain in clot promoting activity until equal to that of the normal euglobulin suspension The degree of dilution required to equalize the activity of the 2 euglobulins is an approximate measure of the qualitative defect of the unknown plasma

Values Obtained Normal plasma euglobulin solutions when prepared from plasma diluted 1 1000 display a two fold increase in clot accelerating activity over that prepared from plasma diluted 1 10 Further dilution of the plasma used to prepare the euglobulin solution produces no greater increase in clot promoting power Hemophilia A plasma euglobulin solutions *prepared from a 1 10 plasma dilution do not accelerate the clotting of normal* plasma to any great extent and are powerless when tested on a substrate of their own plasma However, as the plasma used in the preparation of the solution becomes increasingly more dilute the euglobulins from hemophilic plasma gain markedly in clot promoting power until they function as well as the normal euglobulin prepared from an equivalent dilution, whether *tested on a substrate of normal or hemophilic plasma* The amount of dilution necessary to equalize the activity of the hemophilic euglobulin suspension with that of the normal depends on the severity of the defect

Solutions prepared from plasma which has a quantitative deficiency in the clot promoting euglobulin (e g hemophilia B plasma) cannot regardless of amount of dilution equal normal euglobulin suspensions in clot promoting power whether tested on a substrate of its own plasma or normal plasma

Precautions and Sources of Error Care must be taken to recover all the precipitated euglobulin from the highly diluted specimens The weight of the dried euglobulin precipitate after correction for salt content will serve as a check for complete recovery Incubation of the euglobulin solution with plasma before recalcification results in the loss of activity that may be complete in 30 minutes Therefore *recalcification should follow at once after* addition of the euglobulin solution to the plasma

Discussion Hemophilia A euglobulin prepared from 10 per cent plasma concentration has little clot promoting activity while normal euglobulin prepared from the same concentration has a strong activity As more dilute plasma is used for the preparation of the euglobulins the activity of the *hemophilic euglobulin gradually increases and eventually equals that of* the normal When a highly active normal euglobulin is incubated with citrated normal plasma before recalcification, much of its activity is lost within 30 minutes when incubated with Hemophilia A plasma it loses all activity in 10 minutes The difference between normal and Hemophilia A plasmas in their response to Hemophilia A euglobulin can be eliminated by appropriate *dilution of the plasmas to about 5 per cent* concentration Euglobulin solutions prepared from Hemophilia A plasma at 1 per cent or less concentration accelerate the coagulation of Hemophilia A blood in

Values Obtained PTC activity of normal plasma 900 per cent, PTC activity of normal serum euglobulin 620 per cent PTC activity of normal serum pseudoglobulin 760 per cent Inert material is obtained when fractions are prepared from the serum or plasma of a patient with a deficiency of this component

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7 Thromboplastin Generation Test of Biggs and Douglas[1]

R L MacMILLAN

Object of the Test To measure the intrinsic thromboplastin activity of the blood

Principle Underlying the Test Biggs Douglas and Macfarlane[2] have shown that blood could generate a powerful thromboplastin that was capable of clotting plasma in 8–12 seconds The factors necessary for the production of the thromboplastin are platelets antihemophilic globulin Christmas factor or plasma thromboplastin component (PTC) and calcium Antihemophilic factor is supplied by citrated plasma treated by aluminum hydroxide and serum provides the Christmas factor or PTC factor

Preparation of Reagents (1) *Collection of blood* 9 ml of human venous blood are mixed with 1 ml of 3 8 per cent sodium citrate

(2) *Platelets* The citrated blood is centrifuged at 1500 rpm for ten minutes The platelet rich plasma is pipetted off and centrifuged at 15 000 rpm for five minutes The platelets are deposited on the bottom of the tube The platelet poor plasma is then poured off and kept for use as the substrate The platelets are washed with 5 ml of physiologic saline a wooden applicator stick is used to break up the clumps of platelets The suspension is

for testing Those specimens yielding poor utilization of prothrombin are used

Separation of PTC from plasma Five ml aliquots of platelet poor oxalated plasma are heated in thin walled glass test tubes at 56°C for 5 minutes and cooled immediately to room temperature in an ice bath The coagulated fibrinogen is removed by centrifugation and the supernatant adsorbed with barium sulfate, fifty mgms barium sulfate C P per ml of plasma The button of barium sulfate with its adsorbed PTC is washed twice by dispersion with 5 ml of 0 02 M acetate buffer (pH 5 2) and centrifuged at 3000 rpm for 30 minutes The PTC is eluted two times from the barium sulfate button with 5 per cent sodium citrate in 0 9 per cent NaCl, so that the final volume of eluate equals the original volume of plasma processed The citrate eluate is dialysed against 0 9 per cent NaCl at 4 C for 24 hours The dialysed eluate may either be frozen at −30° or lyophilized

Preparation of PTC from serum Nine volumes of serum from blood allowed to clot for 24 hours in glass test tubes at room temperature are mixed with one volume of 3 2 per cent sodium citrate and allowed to stand at 37°C for 30 minutes to assure neutralization of any thrombin The pH of the serum is reduced to 2 9 by the dropwise addition of 1 N HCl and allowed to stand for 2 hours at 37°C The pH is adjusted to neutrality by the addition dropwise of 1 N NaOH The PTC fraction of the serum is obtained by adsorbing it on barium sulfate (50 mg/ml of serum) at 37°C for 15 minutes The preparation is then centrifuged at 3000 rpm for 30 minutes and the supernatant discarded The PTC is eluted from the barium sulfate button with a quantity of 5 per cent sodium citrate in 0 9 per cent NaCl at 37 C for 15 minutes so that the volume of eluate is equal to one half that of the original volume of serum The barium sulfate is removed by centrifuging at 3000 rpm for 30 minutes and the supernatant eluate is dialyzed against 0 85 per cent NaCl solution for 24 hours The dialyzed eluate is then reconstituted to the original volume of serum processed and either frozen at −30° or lyophilized

Assay and Steps in Procedure To 2 0 ml of the patient's blood is added 0 1 ml of the fraction (plasma or serum) to be tested Prothrombin concentration remaining in the serum after clotting is determined by the two stage method (see page 117)

Calculation The PTC activity is expressed by the percentage that the fraction tested enhances the defective prothrombin utilization of the patient's blood The formula

$$\text{Per cent enhancement} = \frac{\text{Patient's serum prothrombin per cent} \; minus \; \text{Patient's serum prothrombin per cent after fraction added}}{\text{Patient's serum prothrombin per cent after fraction added}}$$

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centrifuged again and the washing is repeated twice Finally, the platelets are resuspended in physiologic saline equal to one third the original volume of plasma

(3) *Antihemophilic globulin* Aluminum hydroxide C$^{\gamma}$ is prepared by the method of Bertho and Grassman[3] as described by Biggs and Macfarlane[4] 0 1 ml of aluminum hydroxide is added to 1 ml of citrated plasma This mixture is incubated at 37°C for 3 minutes and centrifuged The supernatant plasma is diluted 1 in 5 with physiologic saline

(4) *Christmas factor or PTC* Clotted blood is centrifuged and the serum is allowed to stand at room temperature overnight to allow complete disappearance of thrombin, prothrombin and thromboplastin A 1 in 10 dilution with physiologic saline is used in the test

(5) *M/40 calcium chloride solution*

Performance of the Test Immediately before the test 0 1 ml of platelet poor substrate is placed in eight small uniform tubes in a water bath at 37°C An incubation mixture is prepared containing 0 3 ml of platelet suspension, 0 3 ml of aluminum hydroxide plasma and 0 3 ml of serum The mixture is placed in a water bath and 0.3 ml of M/40 calcium chloride solution is added At minute intervals 0 1 ml samples are withdrawn in a graduated Pasteur pipet Another Pasteur pipet containing 0 1 ml of M/40 calcium chloride solution is held in the other hand The contents of both pipets are injected simultaneously into the tube of substrate The time for a clot to appear is recorded Ordinarily samples are taken for a period of 6 to 8 minutes With normal blood, thromboplastin generation reaches its peak in 2 to 4 minutes and clots the substrate in 8 to 10 seconds The mixture may be placed in melting ice to delay deterioration of thromboplastin and a dilution curve prepared from serial dilutions of the mixture with physiologic saline From the curve the clotting time obtained from mixtures of different components may be expressed as concentration of thromboplastin When the concentrations of thromboplastin formed in a mixture of normal components are plotted against minutes of incubation a graph showing normal thromboplastin generation is obtained (fig 1) If Christmas or PTC deficient serum is substituted for normal serum in the mixture defective thromboplastin generation results (fig 1) A similar reaction occurs if plasma from a patient with hemophilia is treated with aluminum hydroxide and is mixed with normal platelets and normal serum

Normal range of values Biggs and Douglas found a wide range of variability between tests made on different days using different reagents This would appear to be a serious objection to the test but in practice the test is used to measure differences in only one component The other components are kept constant This greatly reduces the variability As the difference

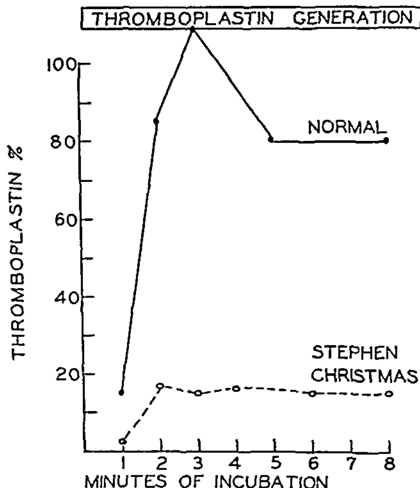


FIG 1 —Thromboplastin Generation Solid Line shows result of mixing normal platelets normal aluminum hydroxide treated plasma normal serum and calcium chloride Broken line (Stephen Christmas) is curve obtained when serum lacking Christmas or PTC factor is substituted for normal serum in the above mixture

between normal and abnormal mixtures is usually large for diagnostic purposes finer criteria of distinction are not necessary

Precautions and Sources of Error Platelets and aluminum hydroxide treated plasma must be freshly prepared every day Serum can be stored in the frozen state for several weeks At the outset of each day the normal re agents are tested If the thromboplastin generated does not clot the sub-

centrifuged again and the washing is repeated twice Finally, the platelets are resuspended in physiologic saline equal to one third the original volume of plasma

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CHAPTER VII

THROMBIN AND PRECURSORS

1 Estimation of Plasma Prothrombin by the One-stage Method

BENJAMIN ALEXANDER

General Considerations and Objectives By chemical isolation and purification Seegers and colleagues[1] have defined the plasma protein component that is convertible to thrombin namely prothrombin Until some other distinguishing physiologic or biochemical property becomes known the determination of prothrombin must be based upon its conversion to thrombin which is then assayed by its ability to clot fibrinogen However within recent years at least two distinct plasma proteins have been discovered which affect the conversion of prothrombin to thrombin by thromboplastin plus calcium [2-6]

Most of the current analytic technics for prothrombin are based upon two methods the one stage procedure of Quick[7] and the two stage method of Warner et al [8] In the one stage method the clotting time of the plasma is measured after optimal amounts of thromboplastin and calcium are provided This the *prothrombin time* is the minimal interval that elapses before a macroscopic fibrin clot results from the conversion of prothrombin to thrombin It reflects among other things the velocity of thrombin formation the amount evolved the velocity of thrombin inactivation by antithrombin the velocity of the thrombin fibrinogen interaction and the concentration of fibrinogen It is clear that the specificity and validity of the one stage method rest upon the assumption that the velocity of prothrombin conversion to thrombin as well as the latter s coagulation of fibrinogen is a measure *solely* of prothrombin concentration

With these considerations in mind it is essential that the investigator clearly recognize his aim in invoking the one stage test If the purpose is to *measure the over all velocity of thrombin elaboration and deposition of a* clot under physiologic conditions the method has far reaching clinical usefulness and few limitations This objective however is to be sharply distinguished from the other goal which is to measure prothrombin specifically In the latter instance the one stage procedure is subject to many limitations Therefore two methods for the one stage determination of

strate in 9 to 11 seconds fresh materials must be prepared This has occurred only occasionally and the fault usually lies with the platelets or the aluminium hydroxide treated plasma The 1 in 5 dilution of aluminum hydroxide treated plasma deteriorates on standing and after one hour a fresh dilution should be prepared Similarly the small tubes of substrate that have been placed in the water bath soon become unreliable It is important that only the exact number of tubes of substrate required for each test are filled and if the room is warm the remainder of the substrate should be kept in melting ice

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opalescent mixture This is subdivided in separate test tubes into 5–10 ml aliquots (the volume in each tube depending upon estimates of anticipated needs of thromboplastin extract for a day's work), and the tubes are kept frozen at −15 to −20°C In the frozen state the extract maintains full potency, giving reproducible results for at least 6 months

Performance of Test The thromboplastin extract is thawed, and 0 1 ml amounts are pipetted into prothrombin time test tubes kept in the water bath Into the tube are added 0 1 ml of oxalated* plasma (1 volume of 0 1 M sodium oxalate to 9 volumes of blood) from a blow-out pipet The mixture is mixed by tapping the test tube with the finger and allowed to stand in the water bath in order to attain the bath temperature 0 1 ml of 0 025 M $CaCl_2$ solution (kept in a 10–20 ml test tube in the water bath) is drawn up with the tuberculin syringe and rapidly ejected into the thromboplastin plasma mixture The stop watch is simultaneously started The mixture is constantly stirred with the nichrome wire loop until the instant of clotting at which time the watch is stopped and the interval from the moment of $CaCl_2$ addition to the moment of gelation recorded All determinations should be done at least in duplicate, and should be in close agreement

Values and Calculations Normal plasma will give a prothrombin time of 11–12 seconds with extracts of human or rabbit brain prepared as described above In this range of prothrombic activity duplicate determinations should agree within ½ second With most commercial brain thromboplastin preparations a somewhat higher prothrombin time is obtained (14–17 seconds) Preparations giving a prothrombin time greater than 17 seconds are generally considered unsatisfactory

Profound reductions in the concentration of prothrombin, of Ac globulin (proaccelerin) of pro-Spca (proconvertin) and of fibrinogen *singly or in any combination* become reflected in a decreased overall prothrombic activity and an associated increase in the prothrombin time (retarded clotting) The degree to which they may be reduced before experimentally significant increases in prothrombin time become evident cannot be stated precisely at present It appears that Ac globulin must be less than 70 per cent of normal before unequivocal elevations in prothrombin time appear [9] fibrinogen must be less than approximately 120 mg per cent [10] and pro-Spca below 50 per cent of normal

Fundamental to the calculation of plasma prothrombic activity from the observed prothrombin time is the curve relating these parameters From figure 1 it is clearly evident that this calculation is greatly influenced by the nature of the diluent employed (for the purpose of deriving the standardization curve) to produce mixtures of varying prothrombic activity

* Citrated (1 vol of 2 5% sodium citrate to 9 vols of blood) plasma may also be used

prothrombic activity will be described (a) the procedure for assaying over all prothrombic activity of whole plasma, and (b) the method for measuring prothrombin more specifically

DETERMINATION OF OVERALL PROTHROMBIC ACTIVITY OF WHOLE PLASMA

Principle The important principles underlying this test have been delineated above It should be further pointed out that the *prothrombin time* of whole plasma is essentially the *recalcification time* of plasma which has been provided with tissue thromboplastic material in excess Under this condition, the recalcification time is greatly shortened by obviating the relatively long interval required for the elaboration of sufficient thromboplastic material from platelets plus plasma factors needed for the prothrombin conversion mechanism

Reagents and Apparatus

(a) Water bath with mechanical stirrer heating elements and thermoregulator maintaining temperature of 37 5°C

(b) Test tube rack and glass tubes 10 x 75 mm

(c) Pipets 1 0 ml calibrated to 0 01 ml subdivision Blow-out pipets calibrated to deliver 0 10 ml

(d) Stop watch nichrome wire loop

(e) Tuberculin syringe and needle

(f) 0 025 M $CaCl_2$ solution

(g) 0 1 M sodium oxalate solution

(h) Thromboplastin Human brain * stripped of pia mater and blood vessels is thoroughly washed with running tap water, and then finely minced with a scissors Two volumes of C P acetone are then added the combination is homogenized for one minute in a Waring Blendor and then filtered The residue is again extracted with fresh acetone in the Waring Blendor for about 15 seconds and filtered The solid material is then spread out on filter paper to dry at room temperature, yielding a powdery non sticky material which is stored in a vacuum dessicator in the refrigerator Full potency is thus maintained for at least a year

Thromboplastin extracts are made by extracting the brain powder with saline as follows 1 5 Gm of the powder are dispersed by shaking in 20 ml of physiologic saline, the combination is incubated at 37 for ½ hour and then centrifuged for 20 minutes at 1200 rpm The supernatant is decanted and the residue is reextracted twice with 20 ml of saline and the three saline extracts (totalling 60 ml) are combined giving a slightly milky

* Brain preparations from other species may be used rabbit brain prepared according to the method of Quick [7] or commercial brain thromboplastin preparations In all instances the potency of the thromboplastin extract being used must be ascertained on a pool of plasma from at least five normal subjects

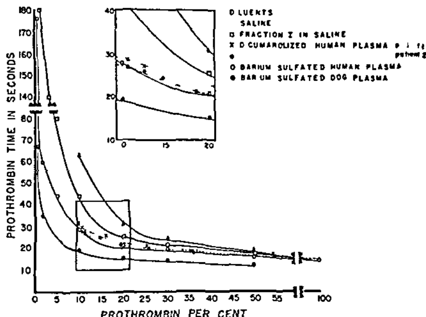


FIG 1—Relation between prothrombin time and prothrombic activity of normal human plasma diluted with various diluents Fraction I (fibrinogen) contained 500 mg of Harvard Fraction I per 100 ml Patients 1 and 2 were subjects with myocardial infarction treated with dicumarol Their plasmas used as diluents contained 7 6 and 9 4 per cent (of normal) prothrombic activity respectively Allowances for these values were made in computing the prothrombin percentage in the dicumarolized normal plasma mixtures (Reprinted by permission of the authors and New England Journal of Medicine [9])

(accelerin and Spca) from their relatively inert plasma precursors (proaccelerin and pro-Spca) occurring very early in the coagulation sequence will shorten the prothrombin time Accordingly inept vessel puncture resulting in contamination of syringe and needle with tissue thromboplastin undue delay in adding anticoagulant or undue exposure of the blood to air bubbles will facilitate elaboration of the accelerators which may lead to artefactual enhancement of prothrombic activity

Also to be considered is the interval between the shedding of the blood and the actual determination Since Ac globulin is relatively labile the plasma should be refrigerated promptly and the determination should be done within a few hours after the blood is obtained It should be noted that repeated freezing and thawing plasma destroys Ac globulin which may result in an abnormally low prothrombic activity

Another possible source of error is the relationship between the con

Moreover, the configuration of the curve indicates that in the range of between 25 and 100+ *per cent (of normal) prothrombic activity* a substantial alteration in activity results in only a very small change in prothrombin time Therefore, conversion of an observed prothrombin time into prothrombic activity in this range is only an approximation, particularly in the higher ranges of activity At lower levels (2–10 per cent of normal), calculation by interpolation on the curve is more accurate

To obtain such a standardization curve prothrombin time determinations *must be made on plasmas with known prothrombic activities varying* between zero and 100 or more per cent of normal Ideally such a curve should be derived from observations on mixtures in varying proportions of whole normal plasma with normal plasma rendered devoid of prothrombic activity but containing normal amounts of all other factors which might influence the prothrombin clotting time At present, we consider $BaSO_4$ adsorbed normal oxalated human plasma the most practical diluent for drawing up the correlation curve

This plasma is devoid of both prothrombin and pro-Spca but contains normal amounts of antihemophilic factor Ac globulin fibrinogen and antithrombin It is prepared as follows to a pool of fresh oxalated plasma from at least 5 normal subjects are added 100 mg $BaSO_4$ (C P , Baker*) per ml , the mixture is shaken every few minutes The mixture is then centrifuged at 3000 rpm for at least 20 minutes and the supernatant plasma is carefully† separated from the sedimented $BaSO_4$ which is discarded The $BaSO_4$ adsorbed plasma should have a prothrombin time of no less than 180 seconds

To this plasma is added in varying proportions, the whole unadsorbed normal plasma and the prothrombin times obtained on each combination are plotted against the per cent of normal plasma in each plasma mixture From this curve (fig 1) which must be established for each batch of thromboplastin *extract the per cent prothrombic activity of a test plasma is interpolated* from its observed prothrombin time

Precautions and Sources of Error Some of the precautions and sources of error have already been alluded to The importance of scrupulous technic in obtaining the blood sample should be emphasized Inhibition of the clotting mechanism as soon as the blood leaves the vein is of the utmost importance for several reasons Obviously partial coagulation will reduce the fibrinogen concentration which may be reflected in an elevated prothrombin time Moreover the formation of the active serum accelerators

* It should be noted that $BaSO_4$ from different chemical houses may vary in its affinity for prothrombin We have found that the Baker Co material has the greatest affinity

† It is important that all the $BaSO_4$ be removed from the supernatant plasma

(c) Thromboplastin $CaCl_2$ mixture Equal parts of freshly thawed human brain thromboplastin extract (prepared as described above) and 0 04 M $CaCl_2$ solution

(d) Human oxalated serum Normal blood is added to thromboplastin (30 volumes blood to 1 volume thromboplastin extract) to induce rapid and complete coagulation the clotted blood is allowed to stand at room temperature for 12 hours the serum is separated and kept at room temperature for another 24–48 hours This assures complete consumption of prothrombin Ac globulin and considerable destruction of whatever pro-Spca had become activated The serum is then oxalated (1 volume of 0 1 M sodium oxalate to 4 volumes serum)

The $BaSO_4$ adsorbed bovine plasma and oxalated human serum are mixed in equal proportions divided into aliquots each of sufficient volume for one day s determinations Stored in the frozen state (−15 to −20 C) the mixture is stable for at least 6 months Repeated thawing and freezing causes deterioration thus a tube containing one aliquot is thawed used for one day s experiments and then discarded

Performance of Test A standardization curve correlating prothrombin time with prothrombin concentration is obtained in a manner similar to that described above in the determination of over all prothrombic activity with certain important differences Because of the much shorter prothrombin times obtained in the presence of small amounts of prothrombin under the conditions of the present test the normal human plasma which is added to the bovine $BaSO_4$ plasma human serum mixture, must first be diluted tenfold with VBOS *

To 0 1 ml of the bovine $BaSO_4$ plasma human serum mixture is added 0 1 ml of this diluted material in a prothrombin time tube in the water bath The mixture is allowed to attain the bath temperature (37 5 C) and 0 2 ml of the thromboplastin-calcium mixture (already brought to 37 5 C) are rapidly added The prothrombin time is then measured in the usual manner The value thus obtained with normal plasma is taken to be equivalent to 100 per cent prothrombin concentration Further serial subdilutions with VBOS of the original 1 to 10 dilution of normal plasma will give values when mixed with the bovine plasma which reflect the correspondingly lower concentrations of prothrombin The standardization curve is thus obtained by plotting the observed prothrombin times against the per cent prothrombin When plotted log/log a linear relationship is obtained except (occasionally) for prothrombin concentrations below 5 per cent of that in normal plasma

* Such dilution has the added advantage of obviating to a large extent if not entirely the possible presence of heparin or other anticoagulants in the test material

centration of anticoagulant and the amount of $CaCl_2$ solution used for recalcification In most instances one volume of 0 1 M sodium oxalate to 9 volumes of blood assures proper proportions However when the blood hematocrit deviates markedly from the normal, as in profound anemia or in polycythemia adequate allowances and correction should be made in the amount of $CaCl_2$ used in the recalcification step

Consideration should also be given to the possible presence of anticoagulants in the plasma, either naturally occurring or added in vivo (heparin etc) That under such circumstances the determination of overall prothrombic activity by the one-stage method would be unreliable, needs no elaboration

One-Stage Method for Specific Prothrombin determination (In the Plasma or Serum)

Principle The validity and specificity of the procedure is predicated upon the assurance that under the conditions of the test the only variable which will determine the prothrombin time is the concentration of prothrombin One must be certain that Ac globulin, pro-Spca, and fibrinogen are present or are provided in optimal amounts, and that other entities such as antithrombic factors are also adequately controlled

A modification of the Owren method[11] seems at present to satisfy these criteria Oxalated bovine plasma deprothrombinated by $BaSO_4$ adsorption is used to provide optimal amounts of Ac globulin and fibrinogen, and human oxalated *serum* is incorporated to provide the Spca system which in the removal of the prothrombin from the bovine plasma has simultaneously been adsorbed The procedure here described is suited to the determination of *residual serum prothrombin* as well as *plasma prothrombin* The results correlate excellently with those obtained with the two stage method

Reagents and Apparatus These consist of those described above The following are also included

(a) $BaSO_4$ adsorbed bovine plasma Beef blood is collected in 0 15 M sodium oxalate (one volume to 9 volumes of blood) The separated plasma is treated with $BaSO_4$ as delineated above except that the adsorption is carried out for 30 instead of 15 minutes

(b) Veronal buffered oxalated saline solution (VBOS)

Veronal buffered isotonic saline (VBIS)	
0 1 M sodium diethylbarbiturate	200 ml
0 1 M HCl	144 ml
0 15 M NaCl	656 ml
Veronal buffered oxalated saline (VBOS)	
0 1 M sodium (or potassium) oxalate	150 ml
VBIS	850 ml

thrombin free serum This does not imply however, that carelessness can be condoned since poor technique and delay in mixing the blood with anticoagulant may result in substantial prothrombin losses, causing erroneously low values

Certain precautions regarding the procurement of bovine plasma are noteworthy The blood is obtained from the major vessels at the slaughter house when the animal is sacrificed by exsanguination From a bucket of blood thus rapidly obtained a proper measured quantity is quickly poured into a receptacle containing the anticoagulant and the mixture is thoroughly stirred Blood occasionally contaminated by esophageal and rumen contents consequent to overzealous slitting of the throat, should be discarded

Other precautions and sources of error are those pertinent to the one stage method for the determination of over all prothrombic activity

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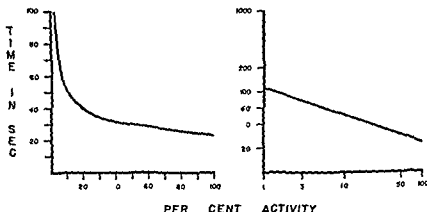


Fig 2 —Typical standardization curve correlating prothrombin time with prothrombin concentration used in one stage method for determining prothrombin specifically Pooled normal human oxalated plasma added in various proportions to a 1 1 mixture of oxalated bovine $BaSO_4$ adsorbed plasma and human oxalated serum 100 per cent activity corresponds to 0 10 ml of a 1 10 dilution of normal plasma with VBOS added to 0 1 ml of the bovine plasma human serum mixture To this are added 0 2 ml of the thromboplastin calcium mixture 50 per cent activity corresponds to 0 1 ml of 1 20 dilution of normal plasma with VBOS etc Curve on right is a log log plot of curve on left

Determination of the prothrombin in a test plasma or serum sample is performed in the same manner including dilution with VBOS The actual prothrombin concentration of the unknown calculated from the observed prothrombin time by interpolation on the standardization curve, is expressed in per cent of normal

A typical standardization curve indicating the prothrombin times representing various prothrombin values is shown in figure 2 Such a curve must be derived for each batch of thromboplastin extract The prothrombin content of individual plasmas from normal subjects varies between 70–120 per cent of that found in a pool from a large number of subjects The accuracy of the method is approximately ±5 per cent

Precautions Sources of Error and Limitations Since most if not all (other than prothrombin) of the factors which affect the prothrombin time are adequately controlled in this method, many of the precautions sources of error and limitations pertinent to the procedure for measuring over all one stage prothrombic activity do not apply here Thus determinations can be made on aged plasma, consideration no longer having to be given to the deterioration of Ac globulin which in this test, is provided by the $BaSO_4$ bovine plasma Similarly less concern need be given to the technique of obtaining the blood sample since activation of the Spca system will not affect the test because Spca is provided by admixture of the human pro-

indefinitely An alternative method of preservation is to place 0 2 Gm of the dry material in small pyrex test tubes cover with 5 ml saline solution (0.85 per cent), and place immediately in the freezing compartment of a refrigerator The material remains fully potent for at least two weeks It requires incubation at 50°C before it is ready for use

Steps in the Procedure Nine volumes of blood obtained by venipuncture are mixed with one volume of 0 1 M sodium oxalate If it is difficult to obtain blood by vein a deep skin cut should be made and the blood allowed to drop into test tube or conical centrifuge tube having a 1 ml graduation and containing 0 1 ml of 0 1 M sodium oxalate The 1 ml sample thus obtained is sufficient for four prothrombin determinations It is convenient to remove plasma by means of a pipet having a rubber bulb from a medicine dropper attached to the end

The prothrombin time is determined by transferring 0 1 ml of oxalated plasma to a small pyrex test tube and then adding 0 1 ml of thromboplastin solution After the tube has been in the water bath for a few seconds 0 1 ml of 0 02 M calcium chloride is blown forcibly into the mixture and the stop watch clicked simultaneously The tube is put in the water bath and shaken lightly A few seconds before the expected clotting time the tube is held toward a distant source of light so that one can see through the tube from below The tube is tilted very gently to permit detecting the incipient web of fibrin which is the end point If the tube is shaken too vigorously the initial fibrin mesh is apt to be broken and so escape detection More time will therefore be required before sufficient fibrin will again be formed to become visible and this introduces an appreciable error

When the prothrombin time is greatly prolonged it is convenient to use a large test tube (25 x 100 mm) filled with water at 37 5 C as a jacket The large tube is fitted with a cork with a hole through which the smaller test tube can be inserted and held in place This device allows continuous observation while the temperature of the reacting medium is maintained constant

Calculation Due to the complex nature of prothrombin it is best to express results in terms of prothrombin activity When the prothrombin activity (in per cent of normal) is plotted against the clotting time a characteristic hyperbolic curve is obtained which is fairly satisfactorily expressed by the equation

$$\begin{array}{c}\text{Prothrombin activity}\\ \text{(Per cent of normal)}\end{array} = \frac{k}{\text{p t} - a}$$

p t = prothrombin time k and a are constants with the values of 303 and 8 7 respectively

2 Estimation of Prothrombin by the One-stage Method of Quick

Adapted by L M TOCANTINS*

Apparatus and Reagents

(1) Pipets 1 ml serologic graduated in tenths and hundredths of a ml The pipets should be cut to 170 mm lengths

(2) Water bath A glass dish 8 to 10 inches in diameter and 4 to 5 inches deep will serve the purpose It should be on a base containing two 25 watt electric light bulbs (These supply sufficient heat to maintain the temperature fairly well at 37°C)

(3) A thermos bottle filled with water at 50°C for incubating the thromboplastin solution

(4) A stop watch

(5) Test tubes pyrex (13 x 100 mm)

(6) Sodium oxalate 0 1 M 1 34 Gm sodium oxalate C P is dissolved in 100 ml of distilled water

(7) Calcium chloride 0 02 M 0 222 Gm anhydrous calcium chloride is dissolved in 100 ml of distilled water

(8) Thromboplastin solution 0 2 Gm of dehydrated rabbit brain is put in a small pyrex test tube covered with 5 ml of 0 85 per cent sodium chloride solution, and mixed by blowing through the suspension with a pipet Triturating the thromboplastin with saline in a mortar should be avoided since this reduces the activity After incubating the solution for 20 minutes at 50°C , it is transferred to the water bath kept at 37°C Agitation of the solution by blowing through it helps to maintain a uniform activity Suspended particles do not interfere with the accuracy of the test but actually aid in the detection of the incipient clot

(9) Preparation of thromboplastin The rabbit brain is freed of all visible blood vessels and then triturated with acetone in a glass mortar The material at first should be mashed and crushed but not ground under a layer of acetone The spent acetone is poured off the brain covered with fresh acetone and the trituration repeated This is continued until the preparation becomes adhesive and flaky It is then ground under fresh acetone until granular The material is filtered by suction and dried at 37 C for 30 minutes By placing the material in glass ampoules evacuating by means of an oil vacuum pump and sealing in a glass flame full potency can be preserved

* With slight modification from a description of the method by A J Quick in the Transactions of the 1st Conference on Blood Clotting and Allied Problems of the Josiah Macy Jr Found 1948 page 170

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Thromboplastin Dry rabbit brain tissue is prepared as follows a rabbit is decapitated by a sharp pair of tin snips The brain tissue and available medulla are removed The free pia mater is removed under running water The tissue is ground to a fine paste in a mortar, spread out on a watch glass and dried in a vacuum dessicator containing calcium chloride The air is removed until a large foamy mass is obtained whereupon the dessicator is maintained at 38–40 C for 24 hours These operations are executed as rapidly as possible The dry tissue is then broken up into a fine powder and stored at 0 C

Preparation of emulsion To 0 1 Gm of the dried tissue is added 5 0 ml of 0.85 per cent NaCl The suspension is stirred and heated at 54–55 C in a water bath for 10 minutes to destroy prothrombin activity The suspension is cooled to 25–26 C 5 0 ml of 0 025 M calcium choride is added to the suspension After being stirred for 4 minutes the mixture is centrifuged for 4 minutes at 1700 rpm The clear or slightly turbid supernatant is carefully removed by pipet

Plasma The blood syringe is rinsed with 0 1 M oxalate solution and 0.2 ml of oxalate is drawn into the cylinder From the dilated vein 1.8 ml blood is drawn and mixed quickly by rotating the syringe The oxalated blood is gently forced from the syringe into a 75 x 10 mm test tube The formed elements are removed by centrifugation at 1700 rpm The plasma is removed by pipet

Assay and Steps in Procedure Into two 75 x 10 mm test tubes 0 1 ml of plasma is added with a serologic pipet graduated to 0 001 ml To one tube is added 0 7 ml of 0 85 per cent NaCl solution to give a concentration of 12 5 per cent To the other is added 1 1 ml of saline to give a concentration of 8.34 per cent The diluted plasmas are mixed thoroughly and placed in a 37 C water bath Into 100 x 12 mm tubes 0 2 ml of the thromboplastin calcium chloride solution is transferred using a 0 2 ml micro blood sugar pipette These tubes are placed in the water bath When the tube contents have reached the bath temperature 0 1 ml of the diluted plasma is added to the tube containing thromboplastin calcium chloride solution The diluted plasma is blown from the pipet and the stop watch started simultaneously The tube is tapped sharply to mix the contents A small stirrer made of #2 nichrome wire is introduced into the tube the stirrer paddle sweeps the test tube from side to side twice per second The end point is that point when the fibrin clot is sufficiently stable to be drawn to one side by the stirrer The variations between duplicate determinations on these diluted plasmas will be one second or less Dilutions of the test plasma are chosen so that the clotting time will fall between that of the two dilutions of the normal plasma A concentration higher than 25 per cent should not

For convenience the following table can be used

Prothrombin Time	Prothrombin Activity in Per cent of Normal
11–12 1/2	100
13 1/2	60
15	50
17	40
19 1/2	30
21 1/2–22	25
24–36	20
37–40	10
55–65	6

Sources of Error Neither the equation nor the table is valid unless a thromboplastin of constant potency is used The prothrombin time obtained on a normal control plasma must fall within the limits of 11 to 13 seconds It is to be emphasized that it is completely erroneous to calculate prothrombin activity by dividing the prothrombin time of the normal by that of the unknown

3 Assay of Prothrombin One-stage Method Using Dilute Plasma (Method of Campbell, Smith, Roberts and Link)

Adapted by R R HOLBURN*

Object of the Method Modifications introduced into the Quick one stage prothrombin time designed to increase reproducibility, and decrease the minimum detectable change in prothrombin

Principle The determination under standard conditions of the time required for the clotting of diluted recalcified plasma in the presence of an excess of thromboplastin

Apparatus and Reagents

Sodium oxalate 1 34 Gm of sodium oxalate C P dissolved in 100 ml of distilled water Solution is 0 1 M

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From Journal of Biological Chemistry 138 1 1941

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4 Estimation of the Blood Prothrombin by the Bedside Method

L B JAQUES

This consists of the determination of the clotting power of the blood to which an excessive amount of thromboplastin has been added The object of the bedside prothrombin test is to provide a *simple rapid* method for the determination of prothrombin times It has been called the accelerated or *actuated clotting time* of the blood

Reagents

Thromboplastin For the Smith bedside method the thromboplastin is prepared from fresh perfused ox or rabbit lung To each 10 Gm of ground lung is added 10 ml of 0 85% NaCl and the mixture is stirred at intervals for several hours and then strained through gauze The fluid is kept at 5°C

For the Schwager-Jaques method commercial acetone-dehydrated brain powder is used The thromboplastin extract is prepared by thoroughly mixing 0 3 Gm of the dried brain with 5 0 ml of physiologic sodium chloride solution and incubating the mixture at 55 C for 15 minutes The coarse particles are removed by slow centrifugation or by spontaneous sedimentation and the milky supernatant liquid obtained The thromboplastin is placed in wide bore capillary tubes (over 2 mm in diameter and 4 inches long) with about 2 inches being drawn into each tube The tubes are sealed in a flame and immediately frozen in the freezing compartment of a refrigerator and kept frozen until used This thromboplastin extract will remain unchanged in activity for as long as six months when preserved in this manner Before use the tube is removed from the refrigerator and warmed in the hand if required for immediate use

Procedure

Smith Bedside Method[1] 0 1 ml thromboplastin is placed in a small tube (75 x 10 mm) which has been marked at 1 0 ml Freshly drawn blood is placed in the tube up to the 1 ml mark The tube is inverted once for complete mixing of the blood and thromboplastin and is then tilted every second or two to observe clotting The test is also carried out under the same conditions using blood from a normal control The clotting activity of the test blood is expressed as a per cent of normal

$$\frac{\text{Clotting Activity}}{(\%\ \text{of normal})} = \frac{\text{Clotting Time of Normal}}{\text{Clotting Time of Patient}} \times 100$$

be used, if necessary, the normal plasma may be further diluted to provide an additional range

Calculation A dilution curve of the standard plasma is made, the points between 5 and 20 per cent falling on a straight line on log log paper Log of the plasma concentration is the ordinate, log of clotting time is the abscissa The clotting time of the test plasma is interpolated on the standard chart and the log of the ordinate read The antilog of the ordinate is calculated, this is the concentration of normal plasma which will give a clotting time equal to that of whatever concentration of test plasma was used Therefore, the relative clotting index or the ratio of the concentration of normal plasma to the concentration of the test plasma is equal to the antilog of the ordinate divided by the per cent of the test plasma that was assayed

Precautions and Sources of Error (1) Dilution curves of all individuals of any species will not be alike, therefore, a normal dilution curve should be set up for each individual for reproducible results (2) By mixing the thromboplastin and calcium together and placing in an empty tube the danger of contamination of stock reagents is minimized (3) The variations in optimum calcium content for individuals might effect the absolute values of the clotting times observed Since the assay method is strictly a differential method, these variations in absolute values will not influence the reliability of the assay If the minimum values for the clotting times are sought they may be realized by making serial dilutions of the calcium chloride solution

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5 Estimation of Prothrombin by the Two-stage Method

R H WAGNER, J B GRAHAM, G D PENICK and K M BRINKHOUS

Object of the Method The basic object of the two-stage prothrombin assay, devised by Warner Brinkhous and Smith[1 2 3] is the specific determination of prothrombin concentration in any solution, regardless of the presence or absence of other clotting accelerators or inhibitors The assay has been used particularly for the determination of prothrombin in plasma, serum and plasma fractions Certain other test procedures as the prothrombin utilization test and a method for measurement of antihemophilic activity are based on the rate of change in prothrombin during clotting as measured by the two-stage assay procedure

Principle Underlying the Assay The assay consists of two stages (1) In the first stage prothrombin is converted to thrombin as rapidly and completely as possible by incubation with optimal amounts of Ca^{++} thromboplastin and known accessory factors (2) In the second stage the thrombin formed at varying incubation times is measured by the speed with which it clots fibrinogen One unit of thrombin is defined as that amount which will cause the formation of a fibrin clot in 15 0 seconds in one ml of a mixture containing the thrombin standardized fibrinogen acacia imidazole buffer and Ca^{++} in an isotonic solution at 28 C One unit of prothrombin is defined as that amount which when fully activated forms one unit of thrombin

As patients with new clotting diseases have appeared and with the preparation of prothrombin in a higher state of purity knowledge of accessory factors needed for the rapid conversion of prothrombin to thrombin in the two stage procedure has increased If the accessory factors are absent in the assay false low values are obtained The discovery of a patient with low Factor V[4] and success in removing AcG (Factor V) from purified prothrombin preparations[5] led to the use of $BaCO_3$-adsorbed beef serum[6] or of Owren s Factor V[7] in the assay More recently it has been discovered that another accelerator factor present in serum is required for rapid and complete conversion of prothrombin to thrombin Mann[8] reported that a serum factor co-thromboplastin (Spca convertin) is needed for the accurate assay of prothrombin in plasma from dicumarolized patients Subsequently Flynn and Coon[9] have shown that spca is essential in the assay of partially purified prothrombin Lewis and Ferguson[10] have described a patient with severe spca deficiency and found that the use of

Schwager, Jaques Method[2] The thromboplastin extract of one capillary tube is expelled into the center of a watch glass Approximately 2 ml of venous blood is drawn into a syringe using a 20 gauge needle Four drops of blood are added from the needle to the thromboplastin on the watch glass The needle is removed from the syringe The watch glass is held on the tips of the fingers and its contents mixed immediately with the needle The needle is passed gently through the mixture until it clots The end point is sharp The time elapsed from addition of the first of the four drops of blood to the end point is the activated clotting time In normal blood the time by this method is 23 to 25 seconds A rapid, neat venous puncture is imperative, as introduction of air bubbles and slow searching for the vein subcutaneously will release tissue juice and the activated time will be abnormally shortened Occasionally, when a fresh thromboplastin extract is prepared the activated times for the normal will be outside this range, in which case the extract is discarded and a fresh one made Range 2 *s d* as per cent of mean = 5 5 per cent This mean should be calculated from results of the test on at least 10 normal adult men and women

The range is expressed best in terms of twice the standard deviation (s d) *as a per cent of the mean Thus, with a mean activated clotting time* of 23 5 seconds twice the standard deviation was found to be ±1 1 seconds which is 5 1 per cent of the mean With a mean time of 64 2 seconds, twice the standard deviation was 3 7 seconds which is 5 5 per cent of the mean Therefore the range expressed as twice the standard deviation is about 5 per cent of the mean Results are reported in seconds or in per cent of normal as in the Smith method already described

Advantages (a) A close correlation exists between the activated clotting time of the blood as performed with this technic and its prothrombin content Therefore an immediate rapid estimate of the blood prothrombin is possible (b) The concentration of calcium and other blood constituents used in the test is close to that of the subject s blood

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beef serum was necessary in the two stage prothrombin assay of the patient s plasma

It remains to be seen whether further advances will increase the number of factors needed

Apparatus and Reagents

(a) *Essential apparatus* (1) Syringes and needles (2) Centrifuge tubes (3) Centrifuge (4) Refrigerator (5) Balance (6) Glass tubes Tubes 10 × 75 mm in size are used in large numbers for the final clotting stage Tubes of larger size are used for storage of reagents (7) 28°C water bath (8) Serologic pipets A supply of 0 2, 1 and 5 ml pipets is needed (9) Stop watch, preferably operated with a foot pedal (10) Additional stop watch or clock with a sweep second hand (11) Visking casing, No-Jax (Visking Corp, Chicago), is used for dialysis (12) Facilities for dialysis at 4°C

(b) *Optional equipment* (1) Freezer Any standard type freezer cabinet capable of maintaining a temperature of −20°C or lower is useful The freezer is used for storage of thermolabile reagents (2) A cold room maintained at a temperature of about 4°C equipped with a centrifuge and dialysis facilities (3) pH meter

(c) *Reagents* Many different technical preparations are satisfactory We describe the ones used in our laboratory Where possible, a list of alternative preparations is given

(1) 0 11 M sodium citrate

(2) 0 11 M sodium oxalate

(3) 0 154 M sodium chloride (normal saline)

(4) Citrated saline one volume 0 11 M sodium citrate plus 19 vol of 0 154 M NaCl

(5) Phenol red 0 1 per cent solution 100 mg phenol red plus 2 8 ml 0 1 N sodium hydroxide and water to dissolve Dilute to 100 ml

(6) Saturated $(NH_4)_2SO_4$ solution 0 01 M potassium oxalate solution is saturated in the cold with $(NH_4)_2SO_4$ and adjusted to pH 7 1–7 2 by the addition of 1 N NH_4OH pH is checked on a 1–10 dilution of the reagent to reduce salt effects

(7) Citrated plasma Obtain blood by venepuncture, using the two syringe method Avoid air bubbles in collection Mix immediately with 0 11 M sodium citrate in a ratio of one part sodium citrate to 8 parts whole blood Centrifuge at about 3 000 g for 20–30 minutes Withdraw the supernatant plasma after determining the hematocrit Keep at 4 C until just before use If the plasma is not to be tested within 1–2 hours after venepuncture, store immediately in freezer

(8) Oxalated plasma Obtain blood as above mix immediately with

0 11 M sodium oxalate in a ratio of one part sodium oxalate to 9 parts whole blood Rest of procedure as in (7) above

(9) Acacia 15 per cent solution Acacia is obtained from Eimer and Amend New York as Gum Arabic USP Select #1 Tears Dissolve with stirring 60 grams acacia in enough hot distilled water to make a 15 per cent solution Centrifuge the liquid while warm and strain the supernatant solution through cheesecloth Adjust the pH to 7.2–7 4 using phenol red as an external indicator Store in the freezer It was found by Ware and Seegers[8] that all lots of acacia tested contained enough Ca^{++} to bring the final calcium concentration within the optimal range when used in the proportions outlined below

(10) Imidazole buffer pH 7.2 Weigh out 1 72 Gm C P imidazole Edcan Laboratories South Norwalk Connecticut Dissolve in approximately 90 ml 0 1 N HCl Adjust to pH 7.2 dilute to 100 ml Store in freezer

(11) Thromboplastins Two different preparations may be used and are equally satisfactory (a) Crude Beef Lung Thromboplastin Five cm cubes of lung are removed from the periphery of fresh beef lungs obtained at the abattoir Large bronchi and blood vessels are removed The cubes are washed thoroughly under tap water to remove as much blood as possible The lung is ground in a grinder with pores 3–5 mm in diameter The ground lung is weighed and placed in an equal weight of saline This mixture is shaken at intervals and extracted at 5 C for 48–72 hours The mixture is then centrifuged and the supernatant fluid is stored in the freezer The concentrated extract is diluted with normal saline to a point at which maximal thrombin yields are obtained in the assay of normal plasma for prothrombin This dilution varies from 5 to 20 fold for different preparations The crude lung thromboplastin preparation should be assayed for prothrombin to rule out the possibility of contamination with this factor (b) Bacto rabbit brain thromboplastin (Difco Labs Detroit) is prepared according to the manufacturer s instructions and diluted 5 to 10 fold with normal saline before use

(12) Fibrinogen All steps in the preparation are carried out at 4 C Oxalated dog plasma is adsorbed for thirty minutes with 100 mg $BaSO_4$ per ml Merck Reagent or Baker and Adamson Reagent $BaSO_4$ are suitable Centrifuge (about 3000 g) for 30 minutes To the supernatant plasma is added one third volume of cold saturated $(NH_4)_2SO_4$ Let stand for 15 minutes Centrifuge at 3 000 g for fifteen minutes Decant supernatant fluid and save for Factor V preparation (see below) Carefully drain tubes containing the fibrinogen precipitate Dissolve the precipitate by gentle agitation in citrated saline using one third the original plasma volume Dialyze against 3 or 4 changes of citrated saline for 2–3 hours After dialysis

beef serum was necessary in the two stage prothrombin assay of the patient's plasma

It remains to be seen whether further advances will increase the number of factors needed

Apparatus and Reagents

(a) *Essential apparatus* (1) Syringes and needles (2) Centrifuge tubes (3) Centrifuge (4) Refrigerator (5) Balance (6) Glass tubes Tubes 10 x 75 mm in size are used in large numbers for the final clotting stage Tubes of larger size are used for storage of reagents (7) 28°C water bath (8) Serologic pipets A supply of 0 2, 1 and 5 ml pipets is needed (9) Stop watch, preferably operated with a foot pedal (10) Additional stop watch or clock with a sweep second hand (11) Visking casing No-Jax (Visking Corp, Chicago), is used for dialysis (12) Facilities for dialysis at 4°C

(b) *Optional equipment* (1) Freezer Any standard type freezer cabinet capable of maintaining a temperature of −20°C or lower is useful The freezer is used for storage of thermolabile reagents (2) A cold room maintained at a temperature of about 4°C equipped with a centrifuge and dialysis facilities (3) pH meter

(c) *Reagents* Many different technical preparations are satisfactory We describe the ones used in our laboratory Where possible a list of alternative preparations is given

(1) 0 11 M sodium citrate

(2) 0 11 M sodium oxalate

(3) 0 154 M sodium chloride (normal saline)

(4) Citrated saline, one volume 0 11 M sodium citrate plus 19 vol of 0 154 M NaCl

(5) Phenol red, 0 1 per cent solution 100 mg phenol red plus 2 8 ml 0 1 N sodium hydroxide and water to dissolve Dilute to 100 ml

(6) Saturated $(NH_4)_2SO_4$ solution 0 01 M potassium oxalate solution is saturated in the cold with $(NH_4)_2SO_4$ and adjusted to pH 7 1–7 2 by the addition of 1 N NH_4OH pH is checked on a 1–10 dilution of the reagent to reduce salt effects

(7) *Citrated plasma Obtain blood by venepuncture using the two* syringe method Avoid air bubbles in collection Mix immediately with 0 11 M sodium citrate in a ratio of one part sodium citrate to 8 parts whole blood Centrifuge at about 3 000 g for 20–30 minutes Withdraw the supernatant plasma after determining the hematocrit Keep at 4 C until just before use If the plasma is not to be tested within 1–2 hours after venepuncture store immediately in freezer

(8) Oxalated plasma Obtain blood as above mix immediately with

(1) Thaw one ml of fibrinogen solution and put 0.1 ml samples in serological tubes in a 28 C water bath Bring other reagents to 28 C

(2) Dilute 0.1 ml of the test sample with a mixture which contains 1 part Factor V and 39 parts citrated saline The aim of this step is to reduce the prothrombin concentration of the sample to approximately 5 units per ml This is the *isometric dilution factor* and it varies with the strength of the sample For normal human plasma this factor is usally 40 to 50

(3) Incubation To three ml of two-stage reaction mixture add one ml of the diluted sample prepared in the preceding step Mix and note the time Incubate mixture in 28 C bath

(4) Testing for thrombin At 30 sec intervals transfer rapidly a 0.4 ml aliquot of the incubation mixture to a tube containing 0.1 ml fibrinogen Simultaneously start the stop watch Keep the contents of the tube in constant agitation Clotting is best observed by holding the tube at an angle in front of a light source so that the lower part of the tube only is strongly illuminated The end point is sharp If clotting does not occur within 25 seconds proceed with the next aliquot

In steps No 3 and 4 above the prothrombin test solution is diluted 5 times This is the *final dilution factor* and is constant

(5) If the first dilution tried (Step 2) gives no clotting time shorter than 18 seconds when tested for thrombin (Step 4) go back to Step 2 and prepare a more concentrated solution, if the fastest clotting time is less than 12 seconds prepare a more dilute solution

TABLE 1 *Relation of Clotting Time to Thrombin Concentration under Standard Conditions*

CT (seconds)	12.0	12.5	13.0	13.5	14.0	14.5	15.0	15.5	16.0	16.5	17.0	17.5	18.0
Thrombin concentration in units per ml	1.34	1.26	1.20	1.14	1.09	1.04	1.00	0.96	0.9?	0.89	0.86	0.82	0.80

Calculations The calculations are illustrated by means of an assay of citrated human plasma

Eight ml whole blood was mixed with one ml citrate solution the cell volume was 3.2 ml Testing of a 1–50 isometric dilution of the citrated plasma gave the following results

Incubation time seconds	30	60	90	120	150
Clotting time seconds	15	14.5	14.1	14.7	15.6

(1) From table 1 14.1 sec is found to be equivalent to 1.09 units of thrombin per ml

bring pH of fibrinogen to pH 7 2 using 1 N HCl or NaOH and phenol red as external indicator Freeze fibrinogen in 1 ml lots

(13) Factor V[11] All steps in the preparation are carried out at 4°C Measure the volume of the supernatant fluid saved from the fibrinogen preparation Add one eighth volume of saturated $(NH_4)_2SO_4$ to bring to one third saturation and let stand for 15 minutes in the cold Centrifuge and discard supernate Dissolve the precipitate in a volume of citrated saline equal to the starting plasma volume (See fibrinogen section above) Dialyze and adjust pH to 7 2, freeze in 5 ml. lots

(14) Beef serum Beef blood from the slaughterhouse is allowed to stand for several hours after clotting The serum at this time is virtually free of prothrombin The serum is removed by centrifugation and frozen

(15) Two stage Reaction Mixture This mixture contains thromboplastin acacia calcium, saline and imidazole buffer It may be prepared with a number of variations It is commercially available in lyophilized form as Bacto Prothrombin 2 Stage Reagent (Difco Labs) This has been manufactured according to the procedure of Ware and Seegers[6] Each ampule is reconstituted by the addition of 10 ml 0 6 per cent sodium chloride For each 2 9 ml of reconstituted mixture add 0 1 ml 1–10 saline dilution of ' prothrombin free ' beef serum (not $BaCO_3$ treated beef serum) The following procedure may be used (a) *Calcium Imidazole Acacia Mixture This mixture is made up in large batches and may be stored indefinitely in the freezer It consists of two volumes of 15 per cent acacia solution three volumes of normal saline and one volume of imidazole buffer*

(b) Reaction Mixture This mixture is made up shortly before use and frozen It consists of two volumes of calcium imidazole acacia mixture, *0 9 volume of dilute thromboplastin and 0 1 volume of beef serum diluted* 1 to 10 with normal saline

Steps in the procedure The assay consists of the following steps *Preliminary dilution of the test sample with Factor V and* citrated saline incubation of the diluted sample with the two-stage reaction mixture, testing of the incubating mixture for thrombin, at intervals, by adding aliquots to fibrinogen and noting the clotting time The shortest clotting time represents the maximum thrombin yield for a given dilution For the purposes of calculation the clotting time must fall in the 12 to 18 second range Therefore the preliminary dilution is varied until the shortest clotting time falls in that range The thrombin concentration in the final mixture is found from the clotting time and table 1 The product of the thrombin concentration times the total dilution is an expression of the total thrombin that could be formed from one ml of native plasma and by definition the prothrombin concentration in units per ml

(1) Thaw one ml of fibrinogen solution and put 0 1 ml samples in serological tubes in a 28°C water bath Bring other reagents to 28 C

(2) Dilute 0 1 ml of the test sample with a mixture which contains 1 part Factor V and 39 parts citrated saline The aim of this step is to reduce the prothrombin concentration of the sample to approximately 5 units per ml This is the *isometric dilution factor* and it varies with the strength of the sample For normal human plasma this factor is usally 40 to 50

(3) Incubation To three ml of two-stage reaction mixture add one ml of the diluted sample prepared in the preceding step Mix and note the time *Incubate mixture in 28 C bath*

(4) Testing for thrombin At 30 sec intervals transfer rapidly a 0 4 ml aliquot of the incubation mixture to a tube containing 0 1 ml fibrinogen Simultaneously start the stop watch Keep the contents of the tube in constant agitation Clotting is best observed by holding the tube at an angle in front of a light source so that the lower part of the tube only is strongly illuminated The end point is sharp If clotting does not occur within 25 seconds proceed with the next aliquot

In steps No 3 and 4 above, the prothrombin test solution is diluted 5 times This is the *final dilution factor* and is constant

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CT (seconds)	12 0	12 5	13 0	13 5	14 0	14 5	15 0	15 5	16 0	16 5	17 0	17 5	18 0
Thrombin concentration in units per ml	1 34	1 26	1 20	1 14	1 09	1 04	1 00	0 96	0 92	0 89	0 86	0 82	0 80

Calculations The calculations are illustrated by means of an assay of citrated human plasma

Eight ml whole blood was mixed with one ml citrate solution the cell volume was 3 2 ml Testing of a 1–50 isometric dilution of the citrated plasma gave the following results

Incubation time seconds	30	60	90	120	150
Clotting time seconds	15	14 5	14 1	14 7	15 6

(1) From table 1 14 1 sec is found to be equivalent to 1 09 units of thrombin per ml

(2) $\frac{\text{Vol citrated plasma}}{\text{Vol native plasma}} = \frac{9.0 - 3.2}{5.8 - 1.0} = \frac{5.8}{4.8}$

$= 1.21$, citrate dilution factor

(3) Thrombin concentration $\times$ citrate dilution factor $\times$ isometric dilution factor $\times$ final dilution factor $= 1.09 \times 1.21 \times 50 \times 5 = 330$ units prothrombin per ml plasma

Values obtained Because of the possible day to day variation in the potency of reagents used, there may be a day to day variation in prothrombin levels in terms of absolute units of prothrombin For this reason in work with plasmas a normal control plasma is assayed at the same time, *and all results are expressed in terms of per cent of the control*

The values found for normal human prothrombin usually fall in the range of 300–360 units per ml. In a series of ten determinations on a sample of human plasma the concentration was found to be 312 units per ml, with a standard deviation of ±13 units In a series of determinations on ten normal adults the average level was 336 units per ml with a standard deviation of ±32 units

Alternative Points of Procedure (a) Armour Bovine Fraction I (The Armour Laboratories, Chicago) is available commercially as a source of fibrinogen It gives approximately one half the units obtained when dog fibrinogen prepared according to the directions in the Reagent section is used (b) Other useful thromboplastins Soluplastin Schieffelin and Co, N Y N Y Ultracentrifuged beef lung thromboplastin This preparation is described by Ware and Seegers [4] (c) Defibrinogenation of plasma to be tested for prothrombin This procedure has been previously described [2, 3] It consists of adding dilute commercial topical thrombin or a fresh serum, rich in thrombin and deficient in prothrombin, to the sample to be tested just before the isometric dilution step (see p 109) After a few minutes the fibrin clot is rolled out the plasma antithrombin quickly inactivates the added thrombin The rest of the procedure is as outlined

Defibrinogenation appears to be a desirable but not an essential step unless the unknown sample contains fibrinogen and very little prothrombin When it is omitted from the procedure a small clot sometimes forms in the incubating mixture If the clot is pipetted along with the thrombin into the fibrinogen, it must not be confused with the end point, which is the formation of a large easily visible clot

Discussion Precautions, and Sources of Error (a) When plasmas or sera are being tested the maximal thrombin yield obtained may be somewhat less than that which would be obtained in an antithrombin free system, since some of the thrombin formed may be inactivated by antithrombin before it participates in the thrombin fibrinogen reaction As a

result when different dilutions of a given sample are tested the more dilute samples containing less antithrombin may give a somewhat higher unitage of prothrombin For most accurate work, the greatest dilution of sample is found which will still fall within the 18 second range By the use of alcohol in the thrombin forming mixture to inhibit the reaction between thrombin and antithrombin Sternberger[12] has devised a two-stage type prothrombin assay which he has shown to be independent of this variable In our experience this precaution is not essential in samples rich in prothrombin (b) The use of Fraction I as fibrinogen gives a low prothrombin unitage Since this means that a more concentrated sample must be used a greater variation in results from the presence of more antithrombin is to be expected in the analysis of plasma (c) Thrombin solutions used to remove fibrinogen from plasma may cause partial inactivation of prothrombin The loss is normally less if fresh serum is used as a source of thrombin (d) Heparin is not a satisfactory anticoagulant for obtaining plasma samples

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6 Preparation and Purification of Prothrombin

J F JOHNSON and W H SEEGERS

The main steps in the method for production of prothrombin consist of precipitation of the prothrombin from plasma at an acid pH followed by adsorption on magnesium hydroxide elution and then fractionation with ammonium sulfate and final isoelectric precipitation

Reagents and Materials

Plasma The blood for this plasma is collected in a special anticoagulant consisting of 1 85 per cent potassium oxalate ($K_2C_2O_4$ $2H_2O$) and 0 5 per cent oxalic acid ($H_2C_2O_4$ $2H_2O$) One part of anticoagulant to nine parts of blood is used After collection the plasma is obtained as soon as possible and either stored in a deep freeze or used at once If human plasma collected in the usual anticoagulant is used the plasma is first dialyzed for one hour against cold water to reduce the anticoagulant concentration

Oxalated saline 0 75 Gm $K_2C_2O_4$ $2H_2O$ is mixed with 0 85 Gm NaCl and dissolved in 100 ml of water Larger amounts of this material can be prepared and kept on hand for use if much prothrombin is to be made

Magnesium hydroxide cream Slowly, and with constant stirring 5 liters of concentrated NH_4OH are added to 20 liters of 20 per cent $MgCl_2$ The precipitate is allowed to settle and is washed several times in the water to remove the ammonia Five hundred Gm of the centrifuge packed $Mg(OH)_2$ is suspended in 1 liter of H_2O A commercially prepared paste Hydro Magma Parte (Dow Chemical Company) may be used instead of the preparation described It is used in the proportion of 1 2 with water

Ammonium Sulfate A saturated solution having a specific gravity of at least 1 26 is prepared by saturation at room temperature

Method The procedure described is a two-day procedure for the processing of 6 liters of plasma The amounts can be less but the time required is about the same for smaller quantities By overlapping four products can be made in five days This is done by starting the second product while finishing the first on the second day, etc

First Day Two large Pfaudler tanks or large metal containers are partially filled with cold tap water to a volume of about 40 l each The plasma should be diluted about 15 times and when 3 l of plasma are used this will be the amount of water necessary Cracked ice is added and the water agitated vertically with a special paddle until a temperature of 0 C is achieved At this point the ice is removed and three liters of plasma that has been strained through saline washed gauze is added to each tank This gives a final plasma water ratio of 1–15 The water and the plasma is

mixed well with the paddle The pH of the solution is adjusted to 5 1 or 5 2 by the addition of cold 1 per cent acetic acid thoroughly mixing the acid with the diluted plasma before checking the pH When all the required acetic acid is added the tanks are covered and allowed to stand for three to four hours to allow the proteins to settle to the bottom The supernatant is then removed by suction being very careful not to disturb the protein layer When as much of the supernatant has been removed as it is possible to get the prothrombin with other materials precipitated is drawn off from the base of the tank

This precipitate is then centrifuged in the cold at 500 to 600 g This can be done in 100 ml centrifuge tubes in a refrigerated centrifuge or in any other device which will accommodate the volume After spinning the supernatant is discarded and the precipitate is placed in a Waring blendor The tubes are washed out with a small amount of oxalated saline and this wash fluid is added to the blendor More oxalated saline is added to a portion of the mixture while in the blendor No more than is necessary to obtain an adequate mixing of the saline and the protein is added The blendor is then used for a period of time long enough to procure complete mixing without foaming and then the suspension is transferred to a beaker in an ice bath while the remainder of the material is treated in like manner A minimal amount of oxalated saline is u ed to clean out the blendor and this is then added to the beaker

A mechanical stirrer is now placed in the beaker and glass pH elec trodes are dipped into the solution With constant stirring 0 1 N NaOH is added slowly until the pH is raised from an initial level of about 5 5 to 6 4 The alkali must be added slowly to prevent local denaturation care being taken to avoid suspension of the material in the foam at the surface All pH determinations require turning off the mechanical stirring motor and being sure that the electrodes are immersed in the liquid

This neutralized solution is now placed in 100 ml centrifuge tubes precooled in an ice bath They are spun in the cold for five minutes at 3 000 to 3 500 g Following this the supernatant liquid is decanted through saline washed gauze into a large beaker To this is added the magnesium hydroxide cream previously prepared and stored in a refrigerator U e 225 ml of $Mg(OH)_2$ to 21 ml of supernatant or proportionate amounts if the quantities are less Thorough mixing of this suspension is essential because at this stage the prothrombin is adsorbed on the magnesium hydroxide particles This is centrifuged at 500 g for 20 minutes to bring down the particles The supernatant is discarded and the white precipitate is transferred to a cooled Waring blendor with about 350 ml of cold physio logical saline that has been used to wash out the centrifuge tubes This is now mixed thoroughly to wash the $Mg(OH)_2$ This mixture is transferred

to the cold 100 ml centrifuge tubes and spun in the cold at about 2,000 g for 5 minutes The supernatant is discarded, then the precipitate is removed using a glass rod to break it up and then washing out with portions of a total volume of 350 ml of cold 0 85 per cent NaCl It is then poured into a Waring blendor and mixed thoroughly Excessive foaming is to be avoided The suspension is then replaced in the centrifuge and spun at 2 500 g for 5 minutes, removed with 0 85 per cent NaCl and stirring rod as before, after discarding the supernatant After this final washing it is blended with about 400 ml of cold 0 85 per cent NaCl A thorough mixing is important at this stage to place the $Mg(OH)_2$ and adsorbed prothrombin in a fluid suspension When complete suspension is obtained, the mixture is transferred to a special pressure bottle

This bottle is a heavy walled 1 liter Seltzer bottle which has been covered with surgical tape as reinforcement to minimize danger of breakage It is fitted with a special pressure cap which can be tightened to prevent the loss of gas when pressure is built up within This cap is attached to a tank of CO_2 by pressure tubing

After the solution has been placed in the bottle the cap is attached and the pressure raised to 40 lb after preliminary flushing twice with carbon dioxide to remove all air The bottle with pressure tube and all is enclosed in a metal protective shell and then placed on a shaker and agitated for 30 minutes After this time the bottle is removed and placed in an ice bath The pressure is maintained until the apparatus has cooled, at which time it is reduced slowly to prevent excessive foaming The mixture is then placed in a metal beaker covered and placed in the refrigerator overnight

Second day The beaker is removed from the refrigerator and the contents strained through 6 layers of oxalated saline washed gauze into a 500 ml graduate by which the volume can be determined To get all the eluate gentle manual squeezing of the gauze is most efficient

A salt and ice bath is prepared to obtain a low temperature of about −10 C and a 2 l beaker well wrapped with gauze or towelling as insulation is placed in the bath which has been similarly equipped with protective wrappings The eluate is poured into the beaker while constant mechanical stirring prevents freezing at the sides of the beaker When the temperature of the solution has reached 0°C saturated ammonium sulfate is added dropwise so that the temperature does not rise A volume of ammonium sulfate is added equal to the volume of the eluate in order to obtain a final concentration of 50 per cent saturation When all of this chemical has been added the beaker is transferred to an ordinary ice bath and then the contents are poured into 100 ml centrifuge tubes precooled in an ice bath

The tubes are centrifuged in the cold at 2 500 g for 15 minutes The supernatant is then decanted into a 2 liter metal beaker The beaker is placed in the salt ice bath as before and stirring resumed When 0 C has been reached more ammonium sulfate equal to the original volume of the eluate is added slowly This should give a final concentration of 67 per cent When all the sulfate has been added the mixture is poured into a precooled glass beaker in an ice bath This is allowed to stand for 20 min utes during which time crystals of magnesium salts are formed which sink to the bottom of the vessel 100 ml centrifuge tubes are filled with the liquid above the crystals, avoiding them as much as possible The tubes are centrifuged at 5 C at 2 500 g for 20 minutes The supernatant is discarded

The beaker containing the rest of the mixture not centrifuged this first time is then emptied of liquid being very careful to obtain the layer of fluid just above the crystals, for it is here that most of the protein is concentrated and as much of it as possible must be removed These tubes are then centrifuged as were the first ones The supernatant is again dis carded and the tubes washed out individually with distilled water This washing should be done carefully by keeping the tubes inverted after pouring off the supernatant A stream of water is directed at the sides of the tube from below through the mouth of the tube The precipitate must not be touched by the water

After washing the tubes are placed in an ice bath The precipitate in the first tube is broken up with a stirring rod and three to five ml of dis tilled water are added This is transferred to the second tube and the process repeated until all the tubes have been rinsed The material from the last tube can be removed quite conveniently with a hypodermic syringe fitted with a 15 gauge needle or similar implement Next the tubes are rinsed in serial fashion with three to five ml of water and the final volume collected in the same manner

The precipitate removed is then dialyzed against de ionized water to remove the ammonium sulfate This may be done with the usual laboratory dialyzing apparatus or modifications thereof We have developed a special apparatus consisting of two disks of DuPont cellophane supported by a cast aluminum frame and the whole rotated in cold deionized water This is more efficient than the usual Visking casing dialysis bag kept in motion in a water bath In either system the water should be changed frequently in order to achieve complete and rapid removal of the ammonium sulfate

A specific resistance of 1 700 ohms or higher should have been reached in about three hours If it is 500 ohms or higher the resistance can be raised by the addition of distilled water or de ionized water until the

desired resistance is attained An equal volume of water will almost double the resistance If the resistance is less than 500 ohms the dialysis will have to be repeated

When the desired resistance is achieved the prothrombin solution is put in a 100 ml beaker which has been placed in an ice bath This is the first step of the isoelectric precipitation of prothrombin making use of the relative insolubility of prothrombin in water solutions at isoelectric point

Dropwise 0 25 per cent HCl is added from a micropipet with constant mechanical stirring until a pH of 5 35 or 5 4 is reached The solution is then centrifuged in a 100 ml centrifuge tube at 2,500 g for 5 minutes to remove the fine cloudy precipitate which contains impurities

The supernatant is placed in a beaker cooled with an ice bath as before and more acid is added in the same manner with constant stirring until the pH is 4 6 Precipitation of prothrombin will give the solution a milky appearance This suspension is transferred to a centrifuge tube and spun at 700 g for three minutes The supernatant is discarded and the sediment is broken up with a stirring rod and about five ml of distilled water added slowly This suspension is then placed in a 50 ml beaker in an ice bath along with approximately five ml of saline used to rinse the centrifuge tube With constant mechanical stirring 0 1 N NaOH is added until the pH is raised to 6 5 or 7 5

The final volume is now transferred to a weighed Erlenmeyer flask by means of a five ml pipet in order to keep track of the volume Three ml of distilled water is used to rinse the beaker the pH electrodes and the stirrer and then added to flask

The final volume is recorded and 0 5 ml is removed for analysis The remainder can be frozen or freeze dried for storage in a dessicator

Careful work and attention to detail should result in a product having approximately 23 000 to 28 000 units of specific activity per mgm of tyrosine and containing 12 000 to 14 000 units per ml of prothrombin Variations above and below this arbitrary standard occur due to the use of different lots of plasma When technical errors are at a minimum there still exists a variation which can only be ascribed to the initial material.

Should the analysis reveal a product of poor quality it can be raised by partial adsorption with barium carbonate This process will raise the specific activity but decrease slightly the total number of units contained due to the adsorption of prothrombin on the barium carbonate with successive exposures to the adsorbent A product can be raised from 26 000 or 27 000 to 29 000 or even higher but further purification is not usual in a product already of 28 000 units or more per mgm tyrosine To use the barium carbonate to adsorb impurities about 20 ml of the prothrombin is shaken with 1 gm of barium carbonate The barium carbonate is re

moved by low speed centrifugation Repeating the adsorption may be tried but it has been found that a single mixing with barium carbonate seems to be as efficient as many in fact more than three or four will lead to loss of activity

Analysis

Prothrombin All analyses of prothrombin products are done by the modified two-stage procedure as described elsewhere (page 105)

Specific Activity The units per mg of tyrosine are determined by means of the Folin Ciocalteau reaction using 0 1 to 0 2 ml of the product From the value obtained by this procedure and the number of units per ml as obtained by the prothrombin analysis the final number of units per mgm of tyrosine can be calculated Units per mgm of dry weight can be found by determining the weight of the product after freeze drying in the tared Erlenmeyer flask

If a freeze drying method is not available the prothrombin can be dried with acetone and stored in a de sicator There will be a loss of activity of the final product by acetone drying and an analysis will have to be done on the product after drying to obtain the final activity before storage This is not true of the freeze dried material Analyses should always be done before analytical use after either procedure because there is a gradual loss of potency in all products during storage

REFERENCE

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7 Estimation of the Rate of Prothrombin Utilization*

J B GRAHAM, R D LANGDELL and
K M BRINKHOUS

Object of the Test This test is designed to furnish an index of the rate of prothrombin conversion to thrombin during clotting The results may

* Investigations leading to this method were supported in part by research grants H 1648 and H 1333 from the National Heart Institute Institutes of Health Public Health Service

be expressed in many ways, but a convenient method is to give the prothrombin half life in minutes The prothrombin conversion is slow in a number of conditions such as hemophilia and thrombocytopenia

Principle of the Test When normal blood clots, prothrombin is transformed into thrombin Because of the inactivation of thrombin by antithrombin, the amount of free thrombin present at any time during clotting is low and is not a reliable index of the efficiency of the clotting reaction By stopping prothrombin conversion of clotting whole blood at time intervals by the addition of sodium citrate and centrifugation, the amount of prothrombin remaining in serum can be measured by the two-stage prothrombin method At the time of actual clotting of fibrinogen, very little prothrombin has been converted to thrombin Thereafter prothrombin is converted to thrombin more rapidly, until in a few hours only traces of prothrombin remain

Reagents and Apparatus The same as for the two-stage method for determination of prothrombin (see page 105) In addition to the items listed for this assay, silicone treated syringes (Dri Film No 9987 General Electric Co) are needed for collection of blood and 10 x 75 mm tubes graduated at the 1 ml mark are required for clotting tubes

Steps in the Procedure (a) The two-syringe method is used for collection of the blood Only blood collected in the second syringe is used in the test A minimum of 10 ml of blood is drawn into a silicone treated syringe (b) One ml of blood is distributed into each of ten 10 x 75 mm clean tubes graduated at the one ml mark (c) Add 0 15 ml 0 11 M sodium citrate solution to each of two tubes at the following intervals 0 20, 40 60 and 120 minutes If the tube has clotted prior to the addition of the citrate solution gently free the clot from the test tube wall with a wooden applicator stick Immediately centrifuge at 3000–5000 g for 5–10 minutes (d) Aspirate supernatant citrated serum (or plasma) with a capillary pipet and attached rubber aspirating bulb Pool the supernatant serum from each pair of tubes (e) Freeze and store each sample Determine the prothrombin content of the samples by the two-stage method

Calculations Values obtained (a) Determine the prothrombin units per ml of each sample In this calculation the citrate dilution factor can be neglected The values thus obtained are low but since the citrate dilution factor is constant for the series the relative values are unaffected The amount of residual prothrombin in each sample is expressed in per cent using the sample citrated at 0 time as 100 per cent (b) Plot the residual prothrombin in per cent against the elapsed time prior to citration using rectangular graph paper By interpolation the time in minutes required for utilization of 50 per cent of the prothrombin is determined This time is termed the prothrombin half life If less than 50 per cent of

the prothrombin is used up during the longest incubation time, the result is expressed merely as a prothrombin half life of >120 minutes

Alternative Procedures (a) Imidazole may be used to buffer the whole blood prior to citration The imidazole buffer is described under the assay of prothrombin by the two-stage method (page 105) If the buffer is used the clotting tubes are graduated at the 1 15 ml mark, 0 15 ml imidazole buffer is placed in the tubes first then 1 0 ml whole blood and tubes are gently mixed Otherwise the procedure is the same as described above (b) The results may be expressed as a prothrombin utilization index [3] or merely as the amount of residual prothrombin in per cent remaining at 60 and 120 minutes

Normal Range of Values For human blood an average of 10 normal subjects gave a value for prothrombin half life of 36 minutes with a range of 31-41 minutes For dog blood the mean value was 28 minutes

Precautions and Sources of Error (a) Care should be taken to avoid air bubbles or undue manipulation in the collection of the blood and its distribution into the clotting tubes Also the tubes should be scrupulously clean care should be taken that they do not become contaminated with silicone which will change the surface property of the glass Variable and non-duplicable results are obtained if these precautions are not observed (b) The clotting tubes after citration should be centrifuged immediately Prothrombin conversion is not stopped completely until all of the serum is expressed from the clot (c) If a severe hypoprothrombinemia exists this test cannot be satisfactorily carried out

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Reagents and Apparatus The same as for the two-stage method for determination of prothrombin (see page 109) In addition to the items listed for this assay silicone treated syringes (Dri Film No 9987 General Electric Co) are needed for collection of blood and 10 x 75 mm tubes graduated at the 1 ml mark are required for clotting tubes

Steps in the Procedure (a) The two-syringe method is used for collection of the blood Only blood collected in the second syringe is used in the test A minimum of 10 ml of blood is drawn into a silicone-treated syringe (b) One ml of blood is distributed into each of ten 10 x 75 mm clean tubes graduated at the one ml mark (c) Add 0 15 ml 0 11 M sodium citrate solution to each of two tubes at the following intervals 0, 20, 40 60 and 120 minutes If the tube has clotted prior to the addition of the citrate solution gently free the clot from the test tube wall with a wooden applicator stick Immediately centrifuge at 3000–5000 g for 5–10 minutes (d) Aspirate supernatant citrated serum (or plasma) with a capillary pipet and attached rubber aspirating bulb Pool the supernatant serum from each pair of tubes (e) Freeze and store each sample Determine the prothrombin content of the samples by the two-stage method

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tively The solution is then allowed to stand at room temperature until no more thrombin forms There may be activation of about 75 per cent of the prothrombin to thrombin Then one may proceed in a variety of ways to obtain thrombin from this mixture

The thrombin in citrate solution may be diluted with water to about 100 ml and precipitated by the addition of 200 ml of saturated ammonium sulfate This should be added slowly so that the temperature of the solution can be kept as near 0 C as possible The ammonium sulfate is added dropwise while the thrombin is in an ice bath and being stirred Centrifugation at 2 500 g in the cold is then used to separate the precipitate which is dissolved in about 10 or 15 ml of water and then dialysed sufficiently against cold water to remove the ammonium sulfate It may be dried from the frozen state There is invariably a loss of activity during the drying process Storage of liquid products at −20 C is also feasible but these frozen products are not as stable as the dried material However in 50 per cent glycerol solution thrombin is remarkably stable and if such solutions are stored in a deep freeze they perhaps represent the most convenient way to use thrombin for most practical purposes

Assay The number of units of thrombin is determined by a standard analysis in which the thrombin is diluted until it gives a 15 second clot with a standardized solution of fibrinogen The titration mixture needed for the analysis follows

Acacia (15% solution)	2 parts
Imidazole buffer (pH 7 2–7 4)	1 part
CaCl (0 70%)	2 parts
NaCl (0 85%)	4 parts

This titration mixture gives the same results as the one used in the prothrombin analysis by the two-stage method For convenience a standard solution of the prothrombin incubation mixture is kept on hand and when a thrombin analysis is to be done this is diluted ½ with 0 85 per cent NaCl Thus there would be 5 ml of saline added to 10 ml of the prothrombin incubation mixture to prepare the thrombin incubation mixture

A standard 1 per cent solution of fibrinogen is then thawed after removing from the deep freeze and is pipetted into serologic tubes for the test 0 1 ml is used in each tube

For a thrombin analysis the dilutions of the thrombin are made in silicone or paraffin lined glassware to prevent the adsorption of thrombin on glass When concentrated solutions of thrombin are handled this adsorption will involve only relatively few molecules and the final concentration will not be affected In dilute solution however the adsorption is easily detected because the thrombin involved by adsorption is an apprecia

8 Preparation, Purification and Assay of Thrombin

J F JOHNSON and W H SEEGERS

Thrombin of the highest quality is obtained by the autocatalytic activation of purified prothrombin in 25 per cent sodium citrate Activation by this means makes it possible to avoid the introduction of calcium inadequately purified Ac globulin and thromboplastin with its multiple components and other unknown factors Thus, the final thrombin will be free of these contaminants

Materials

Prothrombin Prothrombin used as the substrate is prepared in the laboratory by the magnesium hydroxide adsorption method described elsewhere (page 112) It consists essentially of the precipitation of the prothrombin from dilute acidified plasma its adsorption on magnesium hydroxide, elution followed by precipitation with ammonium sulfate and a final isoelectric precipitation from an aqueous solution The products will vary in purity from one preparation to another Moreover the use of proportionately large amounts of magnesium hydroxide gives high yields of prothrombin whereas small quantities of the adsorbent give lower yields and higher purity For some studies one selects the purest obtainable for as the proportion of impurities increase in the substrate the excellence of the thrombin product decreases It is of interest to note in connection with the last statement that prothrombin products which have lost much activity as judged by the usual two-stage method of analysis involving physiological activation can often be transformed to thrombin in sodium citrate solution This would seem to indicate that there are derivatives in the prothrombin which though not detectable by activation with Ac globulin thromboplastin and calcium are capable of becoming thrombin in the autocatalytic reaction

Sodium citrate A 25 per cent solution is used Lower concentrations activate the prothrombin but the yield is much less a 5 per cent solution can produce some thrombin a 10 per cent solution produces more and so on Above 25 to 30 per cent there is no greater production of thrombin and at the higher concentrations there may be some precipitation of the prothrombin due to the strong salt solutions used

Method Purified prothrombin is dissolved in 25 per cent sodium citrate solution so that the final concentration of prothrombin is about 1–1 5 per cent To start the autocatalytic reaction thrombin may be added so that the ratio of thrombin to prothrombin units is about 4 to 100 respec

In practice, if the endpoint varies more than the range of 13 to 17 seconds the test is repeated with another dilution for experience has shown that the table is not reliable for accurate work outside of this range presumably because of personal variations in technic etc, from one technician to another Three determinations are done for each specimen and they should not vary by more than 0 5 second at most These tests should be run after the dilution has been allowed to stand at least two minutes, or they will vary For some reason endpoints taken during the first minute will be too fast and a higher value of thrombin will be obtained After this initial equilibration time the thrombin is stable and will remain so for a matter of several minutes If the dilution tested is not correct and the endpoint not acceptable then a new dilution will have to be made This can be done using the correction table as a rough guide

The test is run at room temperatures if they are not extreme otherwise at 28 C using a water bath

A known control thrombin standard, in 50 per cent glycerol solution should be used to check all determinations as reagents may vary from day to day This standard thrombin solution can be stored in the cold and used many times A standard can also be obtained from the National Institute of Health of the United States Public Health Service

Calculation Calculation of the result is based on the dilutions performed

$$\text{Initial dilution} \times 5 \times \text{correction factor} = \text{thrombin units/ml}$$

Multiplication by 5 is necessary because of the dilution of the sample by the titration mixture and the fibrinogen The correction factor is obtained from the table as given

Precautions When performing the test, it is of the utmost importance to be careful with all the pipets when doing the dilutions and mixing the materials The pipet tips should be wiped off each time and there should be a crisp clearing of the bore every time a measurement is made All pipets are blown out when measurements are made Care must also be taken so no droplets of the solutions adhere to the sides of test tubes and do not actually become mixed with the main solution

Reference

Seegers W H McClaughry R I and Fahey J L Some properties of purified prothrombin and its activation with sodium citrate Blood *5* 421 1950

ble percentage of the total thrombin available Throughout the work described below, silicone or paraffin lined glassware is used whenever dilute solutions of thrombin are manipulated The only exception to this rule is with the final clotting mixtures in which case standard practice ignores the matter of adsorption

In the test, 0 1 ml of the 1 per cent fibrinogen solution is mixed with 0 3 ml of the titration mixture Then 0 1 ml of the diluted thrombin is blown into the fibrinogen solution from a 0 1 ml serologic pipet The end point is determined with the aid of a stop watch started at the instant of mixing the diluted thrombin with the fibrinogen and incubation mixture The endpoint is the same as that for the other clotting tests the first appearance of granularity in the clear solution as it is held to the light However in a thrombin analysis in the absence of thromboplastin the endpoint is more abrupt in its appearance and is not presaged by increasing turbidity, as it is when the thromboplastin is used This granularity is replaced by a solid clot in a few seconds if this is the true endpoint Should the granules persist and no clot form this is a false endpoint and the test should be repeated No incubation is necessary as in the other tests for prothrombin or Ac globulin because the thrombin is already formed and ready to act directly on the fibrinogen

Should the endpoint vary from 15 seconds the following correction table the same as that used for prothrombin analysis should be used to provide the essential correction figure

Time	Corr factor	Time	Corr factor	Time	Corr factor	Time	Corr factor
11 0	1 50	15 0	1 00	19 0	0 75	23 0	0 65
2	1 47	2	0 97	2	0 74	2	0 64
4	1 44	4	0 96	4	0 73	4	0 64
6	1 41	6	0 95	6	0 73	6	0 64
8	1 38	8	0 94	8	0 72	8	0 64
12 0	1 34	16 0	0 92	20 0	0 72	24 0	0 64
2	1 31	2	0 91	2	0 72	2	0 63
4	1 28	4	0 89	4	0 71	4	0 63
6	1 25	6	0 88	6	0 71	6	0 63
8	1 23	8	0 86	8	0 70	8	0 61
13 0	1 20	17 0	0 85	21 0	0 70	25 0	0 60
2	1 17	2	0 84	2	0 69	26 0	0 49
4	1 16	4	0 83	4	0 68	27 0	0 47
6	1 13	6	0 82	6	0 68	28 0	0 44
8	1 12	8	0 81	8	0 68	29 0	0 43
14 0	1 10	18 0	0 80	22 0	0 67	30 0	0 40
2	1 07	2	0 79	2	0 66	31 0	0 38
4	1 05	4	0 77	4	0 66	33 0	0 34
6	1 03	6	0 76	6	0 65	35 0	0 31
8	1 02	8	0 76	8	0 65	37 0	0 28

In practice if the endpoint varies more than the range of 13 to 17 seconds the test is repeated with another dilution for experience has shown that the table is not reliable for accurate work outside of this range presumably because of personal variations in technic etc from one technician to another Three determinations are done for each specimen, and they should not vary by more than 0 5 second at most These tests should be run after the dilution has been allowed to stand at least two minutes or they will vary For some reason endpoints taken during the first minute will be too fast and a higher value of thrombin will be obtained After this initial equilibration time the thrombin is stable and will remain so for a matter of several minutes If the dilution tested is not correct and the endpoint not acceptable then a new dilution will have to be made This can be done using the correction table as a rough guide

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6	1 41	6	0 95	6	0 73	6	0 64
8	1 38	8	0 94	8	0 72	8	0 64
12 0	1 34	16 0	0 92	20 0	0 72	24 0	0 64
2	1 31	2	0 91	2	0 72	2	0 63
4	1 28	4	0 89	4	0 71	4	0 63
6	1 25	6	0 88	6	0 71	6	0 63
8	1 23	8	0 86	8	0 70	8	0 61
13 0	1 20	17 0	0 85	21 0	0 70	25 0	0 60
2	1 17	2	0 84	2	0 69	26 0	0 49
4	1 16	4	0 83	4	0 68	27 0	0 47
6	1 13	6	0 82	6	0 68	28 0	0 44
8	1 12	8	0 81	8	0 68	29 0	0 43
14 0	1 10	18 0	0 80	22 0	0 67	30 0	0 40
2	1 07	2	0 79	2	0 66	31 0	0 38
4	1 05	4	0 77	4	0 66	33 0	0 34
6	1 03	6	0 76	6	0 65	35 0	0 31
8	1 02	8	0 76	8	0 65	37 0	0 28

to 45 C To the warm saline oxalate solution is added 1 2 g acetone-dried human brain powder (prepared from the cerebrum after removing blood vessels) The mixture is inverted and incubated for 30 minutes at 45 C, stirring gently once during the incubation period Avoid excess agitation Remove the larger particles by light centrifugation for a few minutes The opalescent supernatant is relatively stable for a month or more when stored at —10 C Avoid thawing and refreezing which often results in a drop of thromboplastic activity For use in the test one part of the stock thromboplastin is diluted with 4 parts of 0 85 per cent NaCl The diluted thromboplastin is then mixed with an equal volume of 0 05 M calcium chloride and allowed to stand at 37°C for about 30 minutes before use The best results are obtained when the clotting times with normal plasma fall between 40 and 50 seconds It may be necessary to alter the saline dilution of the stock thromboplastin in order to maintain the clotting times in the optimal range

(c) *Standards* Either untreated beef plasma or prothrombin free beef plasma provides a stable source of Ac G for use as a standard Since the Ac G level of beef plasmas may vary standardization should be carried out by comparison with 5–10 normal human plasma samples in the test system described below When kept at —10 C the undiluted beef plasma is stable for months It should be divided into small portions to avoid repeated freezings and thawings Unless there is some reason to suspect deterioration one standardization against normal human plasma is usually sufficient for a given lot of beef plasma

(d) *Preparation of plasma sample to be tested* Blood is collected by a clean venepuncture Nine volumes of blood are mixed immediately with one volume of 3 2 per cent sodium citrate (0 109 M) If much difficulty is experienced with the venepuncture, or if there is the slightest evidence of incipient clotting the sample should be discarded since the formation of even small amounts of serum Ac G will tend to give erroneous results The plasma is separated by centrifugation as soon as possible If the assay can not be done immediately the plasma should be stored in the refrigerator to avoid deterioration of the Ac G At 4 C Ac G is usually stable in citrated plasma for about six hours For the assay one volume of plasma is diluted with nineteen volumes of 0 85 per cent NaCl

If materials other than plasma are to be assayed suitable dilutions in 0 85 per cent NaCl are prepared so that the clotting times in the assay will fall within the range covered by the standard curve Interference by barium citrate calcium or other contaminating ions should be avoided by preliminary dialysis of the sample against 0 02 M sodium citrate The dialyzed sample should then be diluted 1 20 with saline solution before introducing it into the assay system

CHAPTER VIII

ACCESSORY PLASMA OR SERUM COAGULANT FACTORS

1 Estimation of Accelerator Globulin (One-stage Method of Lewis and Ware)

Adapted by R T CARROLL*

Principle—Formation of thrombin in a system of controlled prothrombin thromboplastin and fibrinogen content is directly proportional to the amount of Ac globulin present

Apparatus and Reagents

(*a*) *Substrate* One unit of outdated dried plasma (Lilly) is reconstituted using about two thirds of the saline solution provided and allowed to stand until the Ac G deteriorates (about six days) Since deterioration takes place much more rapidly in oxalate than citrate the reconstituted plasma is divided into lots of 25 ml and dialyzed at room temperature against 0 02 M potassium oxalate in saline for a period of two days The contents of the dialysis bags are then pooled placed in the refrigerator at 4 C and checked daily for prothrombin and Ac G If the prothrombin drops below 60 per cent of normal the material is discarded To follow the drop in Ac G activity a small aliquot of the plasma (5–10 ml) is adjusted to pH 7 4 with 0 01 N hydrochloric acid and then tested by using as a substrate in the system described below The material is considered ready for use when a clotting time of 90–120 seconds is obtained when 0 85 per cent NaCl is substituted for test plasma in the system The substrate is then carefully adjusted to pH 7 4 with HCl and dialyzed in the cold against 0 02 M sodium citrate in physiologic saline since the presence of oxalate *tends to give difficulty with the end point at slow clotting times* Portions of about 5 ml placed in tightly stoppered tubes and stored at —10 C generally prove satisfactory for a period of several months

(*b*) *Thromboplastin calcium solution* Stock thromboplastin 20 ml of 0 85 per cent NaCl containing 0 4 ml of 0 1 M sodium oxalate are warmed

From Proceedings of the Society of Experimental Biology and Medicine 84 640 1953

2 Estimation of Ac-Globulin Activity by the Two-stage Method

J F JOHNSON and W H SEEGERS

Definition Ac globulin probably exists in the circulating blood as an inactive protein and in this state is called *plasma Ac-globulin* With the addition of thrombin or during the clotting of blood this inert form is changed to the active form *serum Ac-globulin* This protein then participates with thromboplastin and other factors in the acceleration of the conversion of prothrombin to thrombin Further action of the thrombin on serum Ac globulin probably causes its inactivation and disappearance from the circulating plasma in very few minutes

$$\text{Plasma Ac globulin} \xrightarrow{\text{Thrombin}} \text{Serum Ac globulin}$$

$$\text{Serum Ac globulin} \xrightarrow{\text{Thrombin}} \text{Inactive Ac globulin}$$

These observations apply to human chicken dog rat, turtle and guinea pig blood In cow rabbit and cat blood Ac globulin remains in the serum and is detectable as serum Ac globulin for long periods of time However, if large amounts of thrombin are added to these serums inactivation of the Ac globulin also results

Principle of Method In order to keep the substrate levels constant and known an initial prothrombin determination must be done before the plasma level of Ac globulin can be found This is done by the two stage method of Warner Brinkhous and Smith (see page 105) An additional modification of the method is used for each specimen as well as the one described by the authors The modification consists of the addition of a diluted beef serum to the diluting physiological saline This serum contains about 90 units of serum Ac globulin per milliliter and has been diluted initially 75 times to supply an adequate amount of the accelerator for the reaction By this addition any deficiency of Ac globulin in the plasma will be corrected and the full activation of all the available prothrombin will be accomplished

Any major difference in the two analyses that is between the modified and the unmodified methods for prothrombin determination indicates that there is a deficiency of Ac globulin This difference called the differential is large when there is a lack of Ac globulin and small when there is sufficient present There is always a slight differential due to the loss of Ac globulin during the initial defibrination of the plasma with thrombin when the thrombin acts on the accelerator as outlined previously It is only in human

Steps in the Procedure The test is carried out in a water bath or a constant temperature block kept at 37°C The substrate and thromboplastin calcium mixture are warmed to 37°C before beginning a series of assays, and may be kept at this temperature for several hours for convenience in running a large series of tests Most consistent results are obtained if the diluted plasma sample is also warmed to 37°C before adding it to the reaction mixture In a clean 10 x 75 mm glass tube, place 0 1 ml substrate add 0 1 ml plasma (diluted 1 20 with 0 85 per cent NaCl), blow in 0 1 ml thromboplastin-calcium mixture mix, and note the time for clot formation after addition of this reagent The Ac G content of the sample is determined by reference to a standard curve

Calculation A standard curve is prepared as follows (a) Determine the clotting times of 5–10 fresh normal human plasma samples (diluted 1 20 with 0 85 per cent NaCl) in the assay system described above The average of these values represents the clotting time of the 100 per cent standard (b) Make a 1 100 dilution in saline of the same normal plasma samples and determine the clotting times Average the values to determine the clotting time of the 20 per cent standard (c) Determine the dilutions of the stock beef plasma which will give the same clotting times This beef plasma may then be used as a reference standard (d) Plot the results on logarithmic paper with per cent standard as the horizontal axis and clotting time in seconds as the vertical axis Since the values between 10 and 100 per cent fall on a straight line when plotted on logarithmic paper, the use of only two points to construct the curve is adequate For Ac G values which lie appreciably above the 100 per cent level a greater dilution of the test material should be employed in order to bring the clotting times within the range covered by the standard curve

The results are expressed in terms of per cent of the normal

Sources of Error (1) Type of anticoagulant used The stability of human plasma Ac G is markedly affected by the type of anticoagulant used Stability is greatest in citrate while appreciable deterioration takes place in a matter of hours in oxalate The presence of heparin and versene also hasten deterioration (2) Failure to allow a reasonably constant interval of incubation in the constant temperature bath or block before picking up the tube to observe the end point may give rise to erratic results (3) When beef plasma is used as the standard, the clotting time of the 20 per cent standard should be determined within a few minutes, since an appreciable drop in activity may occur (4) Freezing and thawing the thromboplastin solution repeatedly (5) Improper collection of plasma as described

2 Estimation of Ac-Globulin Activity by the Two-stage Method

J F JOHNSON and W H SEEGERS

Definition Ac globulin probably exists in the circulating blood as an inactive protein and in this state is called *plasma Ac-globulin* With the addition of thrombin or during the clotting of blood this inert form is changed to the active form *serum Ac-globulin* This protein then participates with thromboplastin and other factors in the acceleration of the conversion of prothrombin to thrombin Further action of the thrombin on serum Ac globulin probably causes its inactivation and disappearance from the circulating plasma in very few minutes

$$\text{Plasma Ac globulin} \xrightarrow{\text{Thrombin}} \text{Serum Ac globulin}$$

$$\text{Serum Ac globulin} \xrightarrow{\text{Thrombin}} \text{Inactive Ac globulin}$$

These observations apply to human chicken dog rat, turtle and guinea pig blood In cow rabbit and cat blood Ac globulin remains in the serum and is detectable as serum Ac globulin for long periods of time However, if large amounts of thrombin are added to these serums, inactivation of the Ac globulin also results

Principle of Method In order to keep the substrate levels constant and known an initial prothrombin determination must be done before the plasma level of Ac globulin can be found This is done by the two stage method of Warner Brinkhous and Smith (see page 105) An additional modification of the method is used for each specimen as well as the one described by the authors The modification consists of the addition of a diluted beef serum to the diluting physiological saline This serum contains about 90 units of serum Ac globulin per milliliter and has been diluted initially 75 times to supply an adequate amount of the accelerator for the reaction By this addition any deficiency of Ac globulin in the plasma will be corrected and the full activation of all the available prothrombin will be accomplished

Any major difference in the two analyses that is between the modified and the unmodified methods for prothrombin determination indicates that there is a deficiency of Ac globulin This difference called the differential is large when there is a lack of Ac globulin and small when there is sufficient present There is always a slight differential due to the loss of Ac globulin during the initial defibrination of the plasma with thrombin when the thrombin acts on the accelerator as outlined previously It is only in human

plasmas and similar specimens where the concentration of Ac globulin is low that this is particularly noticeable In such plasmas, the values are so low that the loss of a few units can be quickly noticed, whereas in the plasmas with higher values the loss of a few units does not affect the acceleration of the conversion of prothrombin to thrombin, and little loss can be detected

As a word of caution, it may be remarked that this differential can be used only as an indication of some deficiency of Ac globulin and will not serve in any but the crudest fashion as a quantitative test

In the determination of Ac globulin a two stage analysis is used By diluting the original plasma many times to slow the conversion of prothrombin to thrombin one can observe the rate of thrombin formation from purified prothrombin in the presence of thromboplastin calcium and other accelerators By its action on a standard solution of fibrinogen, the amount of thrombin formed can be determined at repeated intervals As the amount of thrombin increases the clotting time of thrombin fibrinogen mixture will decrease Each of these determinations can be plotted and a curve representing the rate of thrombin formation can be constructed

The rate of thrombin formation with the other factors controlled will be directly proportional to the Ac globulin content of the plasma By altering the amount of Ac globulin present a family of curves can be obtained Such a group of curves is presented in the Figure 1 On the graph the intervals between the determinations of clotting time are represented on the horizontal axis in minutes The vertical axis represents the clotting time of the thrombin fibrinogen mixture in seconds

By comparison of the curve obtained from analysis of an unknown plasma with such a set of standard curves the level of Ac globulin can be determined for the specimen An example of such a determination shall be given later

By using the incubation mixture to be described further on the test for Ac globulin is made more sensitive particularly in man The sensitivity is increased by the reduction of the amount of calcium, and this will be found necessary when plasmas of low concentration of Ac globulin are used By the increase in sensitivity an increase in the dilutions can be made and thus the possibility of the formation of clot in the reaction tube will be lessened By the dilution, the reactive elements of coagulation are reduced to such a low level that coagulation can take place only at a very slow rate slow enough so that there will be no interference with the thrombin formation that is being measured at regular intervals If the concentration of fibrinogen is high enough in the reaction mixture fibrin formation will take place due to the constantly increasing production of thrombin The situa

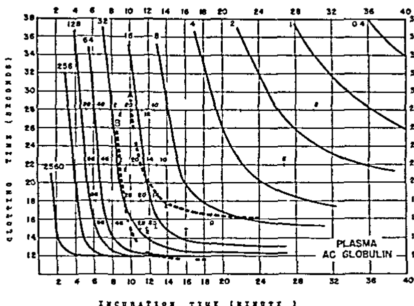


FIG 1

tion is similar to that of a solution of salts seeded with a crystal in which the reaction proceeds at a constantly increasing rate

Bovine plasma is not quite as sensitive to the change in calcium as is human plasma but there will be definite increase in the amount of Ac globulin detected with the reduction of the calcium from the amount present in the incubation mixture used in the two-stage prothrombin test It is *possible to use the same incubation mixture for both determinations if the* above cautions are kept in mind Also to be noted is the fact that this lower concentration of calcium is not the optimum for the complete conversion of prothrombin as in the two-stage prothrombin analysis and compensation for this should be made when the dilutions of the plasma are made in preparation for an Ac globulin analysis The proper evaluation of the plasma can be obtained by performing the two-stage prothrombin analysis using the Ac globulin incubation mixture Values will be lower but they are the ones that will be available in the test for the accelerator

Preparation of plasma and serum for two-stage analysis of Ac-globulin

Collection of the blood When collecting blood for these analyses two syringes should be used The first is to make careful venipuncture and to withdraw a few milliliters of blood then the syringe is disconnected from

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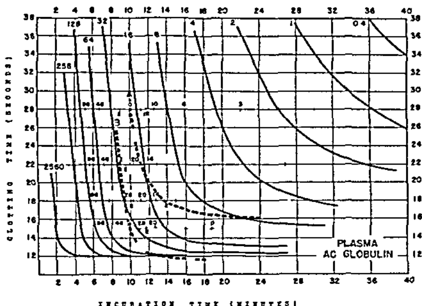


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Preparation of plasma and serum for two-stage analysis of Ac-globulin

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the needle and discarded, following which the second syringe is attached directly to the needle and the blood is drawn into the anticoagulant Blood is then mixed thoroughly and is ready for centrifugation

The choice of anticoagulant is important, particularly when the plasma is to be stored for any length of time It is known that there is a loss in some of the constituents of the clotting systems when blood is collected into 1 85 per cent potassium or other oxalate salts This is particularly true for Ac globulin Therefore, a citrate solution, 3 2 per cent in physiologic saline is a better choice Limited experience with heparin as a preservative, indicates that it can be used, because storage after the use of heparin seems to be satisfactory

When sodium citrate is used, one volume of the anticoagulant is added to nine volumes of blood if potassium oxalate is employed seven volumes of blood are added to one of the oxalate

The blood should be centrifuged as soon as is convenient after drawing but generally this should not exceed one half hour, although a longer delay is allowable, providing that the blood can be kept cool If allowed to stand at room temperature there may be considerable loss of Ac globulin Centrifugation should be carried out at about 2000 g for about 20 minutes or longer in order to obtain adequate and complete separation of the plasma (Some blood coagulation tests are profoundly influenced by the kind of centrifugation) If there is a slight amount of hemolysis after centrifugation it can be disregarded Experience has shown that this will be of little importance in the evaluation of the clotting elements measured by the techniques described

Separation and Preservation of Plasma After separation of the plasma, the analysis should be carried out as soon as possible If however this is not convenient it can be stored for a few weeks in a deep freeze (minus 10-20° F) until the work can be done Storage at these temperatures will maintain the plasma values for about a month Longer storage is discouraged due to the gradual loss of potency of some plasma components Thawing and refreezing manipulations should be kept at a minimum since this procedure is detrimental to the proteins in the plasma During the actual analysis the plasma is best kept in an ice bath when not being manipulated this will insure preservation of the plasma to the greatest degree

To thaw plasma that has been frozen it is best placed in the water bath at 28 C for about 10 minutes with occasional agitation Even with this caution there may be some material in the plasma that will not dissolve This probably represents denatured protein that will go into solution with further heating or can be removed with a cotton swab on an applicator stick Thawing at higher temperatures is acceptable if care is taken to prevent overheating

Separation and Preservation of Serum Blood which is to be allowed to clot for the formation of serum can be drawn directly into a syringe and then transferred to a suitable tube for the formation of the clot The tube is then placed in the ice box and full retraction is allowed to take place This is usually complete after 2 hours At this time the tube is removed from the ice box and centrifuged at 2000 g for 10 to 20 minutes Serum can be preserved in the deep freeze (minus 10–20 F) for a considerable length of time

Preparation of Reagents

Thromboplastin A satisfactory preparation of thromboplastin for use in the two-stage analyses of prothrombin and Ac globulin may be made as follows Bovine lung obtained at the slaughter house is dissected free of the trachea bronchi and the major vessels This is a gross dissection the smaller vessels and bronchi remain in the lung The lung is then ground through a domestic meat grinder and the ground material mixed with an equal amount of 0 85 per cent NaCl This mixture is then reground to insure complete mixing The resulting suspension is then placed in the refrigerator and extracted for the next 24 hours Occasional stirring is necessary during this time This is called the first extract The next day the first extract is strained through gauze and discarded The remaining crude homogenate is then mixed with another equal volume of 0 85 per cent NaCl and replaced in the refrigerator for 48 hours Occasional stirring as before will be necessary The second extract is then strained as before and then passed through a hand operated homogenizer (Central Scientific Co , Chicago Ill) At this stage it can be used for analyses Phenol is added to a final concentration of 0 5 per cent to prevent bacterial growth

The first extract is discarded because it will contain inhibitors of the conversion of prothrombin to thrombin as well as lacking in thromboplastic activity The second extract is considered to be purer and relatively free from antithrombic material Third and fourth extracts have been tried and are low in thromboplastic activity

Before the second extract is mixed with phenol it can be frozen and will remain stable for a long time For use it is thawed and rehomogenized because freezing seems to break the suspension After mixing with phenol the thromboplastin should not be frozen Apparently phenol disturbs the protein suspension and subsequent freezing will alter the proteins to such an extent that the material is no longer suitable for use

If the final lung suspension tends to flocculate or if there is a heavy precipitate or particles present in the final solution it can be centrifuged lightly at not over 1000 g for about 5 or 10 minutes This removes the

gross particles and does no harm to the thromboplastic preparation Re homogenation can be done at any time after the preparation has been made

Testing of the material is done by using it in the standard two-stage analysis for prothrombin using a known plasma as the substrate It will usually be found necessary to dilute the thromboplastin to the ideal value A 1 4 dilution (before placing in the final reaction mixture) is commonly used, one part thromboplastin to four parts of 0 85 per cent NaCl A less active solution may require a lower dilution and the reverse for a relatively active preparation

Acacia and Calcium Acacia is necessary in the incubation mixture to provide the proper colloidal environment for the reaction to progress In many respects it is a technical annoyance, but there is no easy way to dispense with it without throwing modern work out of gear with previously published data The type of acacia used is important only in that the amount of calcium contained must be known We have obtained excellent results using crude acacia which has been analyzed and found to contain 0 68 per cent calcium Purified acacia has been found to be no better than the crude The crude acacia should be ground to a granular state and then dissolved in 0 85 per cent NaCl This procedure is slow and constant mechanical stirring is the best to obtain suspension of the material After the acacia has been dissolved it should be lightly centrifuged at about 2000 g for 5 minutes in order to remove the insoluble debris The material is then strained through 2 layers of gauze to remove particles that are not precipitated by the centrifugation It is then ready for use

A 15 per cent stock solution is prepared This concentration will provide the proper amount of calcium for the conversion of prothrombin to thrombin under the conditions of the reaction Since the amounts of calcium will vary from one batch of acacia to the next the calcium will have to be determined with each new supply If any adjustments are required to obtain the final appropriate concentration of 0 70 per cent calcium $CaCl_2$ is added until this is reached By keeping a large supply of acacia on hand in which the calcium content is known these determinations will be avoided

When this acacia is used in the Ac globulin analysis there will be when it is mixed with the thromboplastin buffer and 0.85 per cent NaCl the proper calcium concentration for the optimum performance of the test The final concentration in the complete mixture has been determined as about 30 milligrams per cent expressed as calcium In prothrombin analysis the acacia solution in the incubation mixture will have to be strengthened with $CaCl_2$ until a final value of 65 milligrams per cent is obtained More calcium is required for the prothrombin assay then in the Ac globulin determination

Imidazole Buffer This is prepared by dissolving 1 75 grams of imidazole (Edcan Laboratory South Norwalk, Conn) in 90 ml of 0 1 N HCl and then diluted to 100 ml volume with distilled water The pH can be adjusted with a few drops of strong hydrochloric acid or sodium hydroxide if it is not 7.2 or 7 4 which is the optimal range for the determinations and use of this buffer

Fibrinogen This protein prepared by the method of Ware, Guest and Seegers (page 157) is diluted to a 1 per cent solution with physiologic saline and should contain 10 per cent imidazole buffer by volume It is stored in the deep freeze and thawed as needed Care should be exercised during the thawing rapid thawing usually being the most de irable Slow thawing of the fibrinogen allows denaturation of the protein, which results in a reduction of the final concentration of the fibrinogen as well as being the source of insoluble material that will make clear definition of the endpoint difficult If a method of rapid thawing is followed there will be little denatured material remaining Any of the altered material which does remain after the thawing can be removed with a small cotton swab on the end of an applicator stick Different solutions of the fibrinogen will behave differently and the treatment should be altered according to each Change in the reactivity of this protein constitute one of the main sources of error in the determinations and infinite care is necessary

Steps in the Procedure First step *Dilution of the Prothrombin and the Ac-globulin* The dilution necessary for human plasma should be greater than 800 times for accurate curves to be obtained on the interpolation chart used in the calculation of the concentration Lower concentrations can be used but there are several disadvantages One of these disadvantages is that with the more concentrated solutions of plasma there may be sufficient fibrinogen present as well as other elements of the blood clotting mechanism to cause coagulation in the reaction tube during the incubation period There is no way to avoid this except by dilution since the fibrinogen cannot be removed from the solution by any way known without altering the Ac globulin or the other ingredients of the mixture

Another disadvantage is that with the increased concentration of the plasma there is an increase also in the plasma antithrombin and this may destroy thrombin at a rapidly increasing rate as the reaction produces more thrombin from the prothrombin The destruction of thrombin resulting will manifest itself by a poorly shaped curve on the interpolation chart with a marked rise from the base line as the reaction proceeds This undesirable effect can be avoided by making the proper dilution of the plasma which will be a dilution large enough so that the effect will be so small that accuracy can be maintained

This dilution of the plasma 800 times or more may be done in one of

two steps In the former approach, a large amount of purified diluted pro thrombin must be used while the second method will be more suitable when there is the desire to save materials In the author's hands the second method is the better and will be described

In designing the method of Ac globulin analysis, Ware and Seegers chose a prothrombin concentration of 1 34 units per ml which would give, on complete conversion to thrombin a 12 second clotting time The reason for this figure being chosen is that it shows on the interpolation chart the greatest change in value in the 13 to 17 second range Examples and more complete elucidation of this will be shown later when the calculation of the results is explained

In order to obtain this level of prothrombin (1 34 units per ml) in the final solutions both the original plasma and the purified prothrombin used must be diluted to 6 7 units per ml This value is obtained when we consider that in the two-stage prothrombin test, in the conversion and thrombin measurement steps there is a dilution factor of 5, and therefore the concentration of the final prothrombin is 5 × 1.34 or 6 7 units per ml The prothrombin level of the original plasma should be determined and then diluted to the desired level with physiological NaCl solution and used for the test at that level After this dilution, the plasma must be further diluted with purified prothrombin that has been previously adjusted to the desired 6 7 units per ml This second step yields the final dilution of 800 or over

The dilution of the purified prothrombin is made with physiological NaCl solution The prothrombin used is prepared from bovine plasma, heated at 54°C for 1 hour and then acetone dried The heating destroys any Ac globulin that may be present For ease in handling small amounts of this product can be dissolved in physiological NaCl solution and then placed in the deep freeze to await thawing at the convenience of the investigator

Products that have been freeze-dried are not as satisfactory for use in this analysis After such treatment there is a greater tendency toward the more rapid formation of thrombin than the acetone dried product and thus a reduction in stability When choosing a product for use one with highest practicable specific activity and purity should be selected because there is a great loss in both when the product is heated and dried The final powder should be put into solution at about 670 units per ml for convenient handling in making the dilution necessary for use in the Ac globulin analysis This concentrated solution is then frozen Thawing is best done rapidly in a water bath care being taken that the prothrombin does not become heated and thus lose some of its activity After thawing the concentrated prothrombin should be kept in an ice bath With these precautions the solution should remain active and stable for several hours However after

dilution the material will lose activity in a shorter time and only as much as is needed for the work at hand should be prepared

Even with careful preparation prothrombin that is stored in the freezer at −5°C gradually loses its strength until a plateau is reached below which the level usually does not drop further for appreciable lengths of time

There appears with the loss of the prothrombin an accelerator of prothrombin conversion which seems to act in a similar manner as Ac globulin It does not substitute for Ac globulin although it acts in similar manner This material appears in a few days in the dissolved prothrombin but not in the dried powder For this reason only small amounts of the powder should be dissolved at any time and the prothrombin thus prepared used fairly quickly When Ac globulin determinations are done with this accelerator as a contaminant of the purified prothrombin extremely high values are obtained When such occurs the prothrombin should be discarded and a new preparation made The use of a daily bovine plasma control enables one to guard against this potential pitfall

With the diluted plasma and the diluted prothrombin on hand the final dilutions of the plasma can be made For example if a final dilution of 1 800 is wanted for the plasma which contains about 300 units per ml of prothrombin the first dilution should be 1 45 This yields plasma with a concentration of 6 7 units per ml This dilution is made with physiologic NaCl solution and is done by placing 0 1 ml of the plasma in 4 4 ml of the solution In these dilutions as is the general practice the pipet is washed out by sucking up the diluent several times Then 1 0 ml of this solution is placed in 3 4 ml of the dilute purified prothrombin previously made up to 6 7 units per ml Thus a final dilution of 198 is achieved or 45 × 4 4 When this is further diluted with the incubation mixture in a ratio of 4 1 the final concentration of the plasma will be 4 × 198 or 792

This method of dilution is designed to be conservative of materials and will be the best in most instances Other methods of dilution are perfectly acceptable if the primary requirement that the concentration of the prothrombin is 6 7 units per ml in the final solution is met A thorough understanding of these dilutions is very important and necessary before any familiarity with the analysis can be claimed

Second step *Incubation Mixture* This incubation mixture providing the other accelerators of the activation of prothrombin to thrombin is composed of tissue thromboplastin acacia imidazole buffer and physiological NaCl solution It is prepared by mixing diluted thromboplastin with a prepared mixture of the other ingredients

The dilution of the thromboplastin is 1 5 with physiologic saline This dilution is one that has been determined from experience as the most

satisfactory for use in the test, and after preparation of the thromboplastin from beef lung, *it is diluted so that this five times dilution gives the proper* answer in the standardization analysis

The remaining materials composing the incubation mixture, are added to the thromboplastin in a 2 1 ratio, one part thromboplastin and two of the mixture as follows

Acacia	2 parts (15% solution in 0 85% NaCl)
Imidazole buffer	1 part (pH 7 25)
0 85% NaCl	3 parts

These materials are kept in the refrigerator all mixed, and a small amount of the mixture is used with the thromboplastin when an analysis is to be done A convenient proportion will be 5 ml of the diluted thromboplastin (1 0 ml thromboplastin to 4 0 ml of 0 85 per cent NaCl) and 10 0 ml of the incubation mixture composed of the acacia buffer and 0 85 per cent NaCl in the proportions described above This final solution is placed in a water bath and kept at 28°C as it is being used There is a *tendency* for the thromboplastin to settle and agitation may be necessary

Third step *Activation* To 2 1 ml of the reaction mixture containing thromboplastin and the incubation mixture is added 0 7 ml of the diluted plasma containing 6 7 units per ml of prothrombin and diluted for its Ac globulin content The mixture is placed in the water bath at 28 C and a stop watch is started at this time because this is the time when incubation begins, and prothrombin becomes activated

Fourth step *Clotting of Fibrinogen to Measure the Thrombin Concentration* At measured intervals usually two minutes apart 0 3 ml of the conversion mixture is removed and added to 0 075 ml of fibrinogen previously pipetted into small glass 12 by 75 mm tubes The clotting endpoint, the same as that of the two stage test for prothrombin is determined accurately by means of a second stop watch started at the instant of adding the conversion mixture to the fibrinogen

The endpoint consists in seeing the first formation of definite granules in the solution observed at room temperature There is invariably a cloudiness developed in the previously clear solution as the endpoint is neared This is followed very shortly by the granularity If this is the true endpoint the granules are followed by the formation of fibrin strands and a definite clot in a few seconds A false endpoint due often to variations in the acacia is not followed by a clot, but usually remains granular for seconds

Calculation The plasma Ac globulin unit may be defined as that amount of plasma Ac globulin which when diluted 1 000 times will convert a standard solution of prothrombin (1 34 units per ml) to thrombin under the conditions of the test, at the rate equivalent to line 1 in figure 1

The actual measurement of Ac globulin concentration in the original plasma requires (a) interpolation from the standard curves of Ac globulin activity, (b) correction for dilution, and (c) expression in terms of the control sample To elaborate

(a) Interpolation from the standard curves If the procedure has been carried out properly the curve constructed by connecting the points obtained by plotting the incubation time (horizontal axis in minutes) vs clotting time (vertical axis in seconds) will have the same contour as the curves in figure 1 The data may read as in the following example

Incubation time (min)	10	11	12	13	14	16	20	24
Clotting time (sec)	34	26	18 3	16	15	13 8	13 8	13 2

When these results are plotted on a chart such as in figure 1, they will be found to fall on the line labeled 16 Therefore by definition the conversion mixture contained 16 one thousands of a unit of plasma Ac globulin

(b) Correction for dilution The dilution preceding the activation is stated in steps 1 and 3 (page 133) of this method and will be of the order of 800 as was calculated before The dilution of the thrombin that occurs in the mixing with the fibrinogen is not a factor in the calculation because Ac globulin exerts its effect in the conversion of prothrombin to thrombin and only the dilutions to this stage will affect the final activity of the Ac globulin

If we use the figure from the dilution example and the figure obtained from the example above the final calculation is

$$\frac{16}{1\,000} \times 192 \times 4 = 12.8 \text{ units per ml of the original plasma sample}$$

(c) Relation to the control The Ac globulin test measures a dynamic process and for this reason is very sensitive to variations in the reagent activity Therefore when an unknown Ac globulin sample is to be analyzed a control should be done under the identical conditions and with the same reagents This should be done simultaneously or within an hour of the determination of the unknown plasma sample The normal control should be a bovine specimen in which the values are known to be stable

If the control serum is needed for a serum Ac globulin source bovine blood is collected as before but no anticoagulant is added The blood is then allowed to clot for about two hours and the serum is separated from the cells by centrifugation This serum is mixed with barium carbonate on which prothrombin is preferentially adsorbed followed by removal of the carbonate by centrifugation until clear serum is obtained

The resulting serum will be practically prothrombin free and a potent

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Fourth step *Clotting of Fibrinogen to Measure the Thrombin Concentration* At measured intervals usually two minutes apart 0.3 ml of the conversion mixture is removed and added to 0 075 ml of fibrinogen previously pipetted into small glass 12 by 75 mm tubes The clotting endpoint the same as that of the two-stage test for prothrombin is determined accurately by means of a second stop watch, started at the instant of adding the conversion mixture to the fibrinogen

The endpoint consists in seeing the first formation of definite granules in the solution observed at room temperature There is invariably a cloudiness developed in the previously clear solution as the endpoint is neared This is followed very shortly by the granularity If this is the true endpoint the granules are followed by the formation of fibrin strands and a definite clot in a few seconds A false endpoint due often to variations in the acacia is not followed by a clot but usually remains granular for seconds

Calculations The plasma Ac globulin unit may be defined as that amount of plasma Ac globulin, which when diluted 1 000 times, will convert a standard solution of prothrombin (1 34 units per ml) to thrombin under the conditions of the test, at the rate equivalent to line 1 in figure 1

Reducing the Ac globulin further continues to reduce the final thrombin yield

Occasionally the form of the experimental Ac globulin curve does not fit into the contours imposed by the interpolation chart (fig 1) In such *instances it may cross one of the curves which are a part of the interpolation* chart Often the defect is of the nature illustrated by line A of figure 1 crossing to the less concentrated side and leveling off shallow Such a pattern may be caused by a deficiency of prothrombin in the conversion mixture the presence of serum type Ac globulin in the analytic sample low fibrinogen activity abnormal colloid forms or calcium concentration Any of the rest of the reagents in the solutions may be suspected as well although those mentioned will be the most likely sources of the trouble Canine plasma gives this sort of curve normally and they are most difficult to interpret on this account

Conversely, a poor curve of the opposite form may pass as does line B of figure 1 to higher values leveling off in the region below that expected from the location of the upper portion of the curve perhaps even with endpoints that are below the 12 0 second line Excessive amounts of prothrombin or hypersensitive fibrinogen are usually responsible for this type of curve Should the curve remain abnormal after checking the prothrombin activity of the plasma the diluted purified prothrombin and the fibrinogen then the other materials must be checked as above

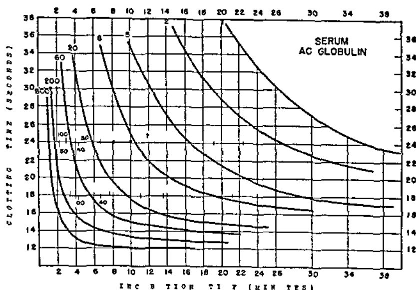


FIG 2

source of serum Ac globulin It is stable and can be stored in the same manner as the control plasma

Stock supplies of these controls are kept in the deep freeze and thawed when they are needed By the use of this bovine control, any change in the reagents can be detected, although it is desirable to use a control of the same species as the unknown, if this is possible The control can be collected at the same time as the specimen for analysis or it can be frozen plasma that is of reasonably recent preparation In some species the content of the Ac globulin falls after the plasma has been placed in the deep freeze and stored for more than a short length of time

Bovine material has a constant value of about 120 units per ml and this value is used to determine the factor necessary to correct the values obtained if there is any deviation from this A simple proportion is used For human plasma Ac globulin values the range is 13 to 17 units per ml and 100 per cent has been placed at 15 units This is an arbitrary figure Values obtained are expressed either as per cent of normal or in the actual units obtained from the determination corrected for the difference shown by the control

For example, in the specimen that we used above the answer was 12 8 units per ml If at the same time we had found that bovine control sample gives us 103 units per ml, the proportion would be as follows

$$12\ 8 \times \frac{120}{103} = 15 \text{ units per ml for the specimen}$$

Other determinations done with the same reagents should be subjected to a similar correction

Precautions and Sources of Error A unit of thrombin is by definition that amount which will clot 1 ml of standard fibrinogen solution in 15 seconds, under standard conditions Clotting times between 13 and 17 seconds have been shown to relate the thrombin concentration most accurately One unit of prothrombin produces one unit of thrombin when fully converted In this test the amount of prothrombin 1 34 units per ml, is such that full advantage is taken of the 13 to 17 second range of end points As a result all curves indicating Ac globulin concentration down to line # 12 of figure 1 have the principal change of direction from vertical to horizontal in the 13 to 18 second range

Although the prothrombin concentration is arranged so the complete conversion furnished 1 34 units per ml, the actual final thrombin yield in the Ac globulin test is dependent on the Ac globulin present Comparison of the curves # 64 and # 32 in figure 1 show that the former ultimately gives 12 0 second clotting whereas the latter only reaches 12 8 seconds

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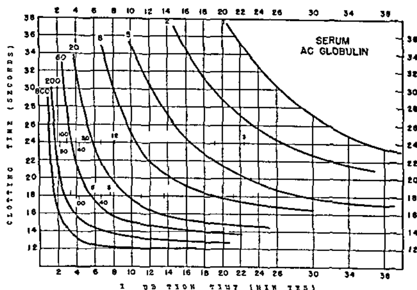


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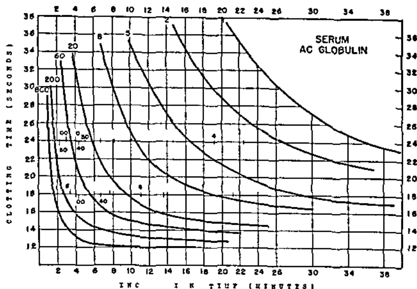


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3 Determination of Spca (Convertin, Factor VII)

BENJAMIN ALEXANDER

General Considerations, Definitions Terminology It is now generally agreed that serum derived from blood which undergoes spontaneous coagulation contains an entity which accelerates or otherwise activates the conversion of prothrombin to thrombin by thromboplastin and calcium [1] Distinct from prothrombin, thrombin Ac globulin antihemophilic factor and other known coagulation factors its deficiency results in a hemorrhagic diathesis despite normalcy of all other clotting factors [2] More recently it has been shown that the prothrombin in plasma or separated from plasma, which has been rendered devoid of this material is at best convertible to thrombin extremely slowly by thromboplastin plus calcium [3]

The substance originally labelled the serum prothrombin conversion accelerator (Spca), arises from its relatively inert plasma precursor, pro-Spca during coagulation This transformation can also be demonstrated in vitro in an isolated system containing thromboplastin and calcium plus plasma or serum fractions which are devoid of prothrombin thrombin, and Ac globulin [4]

The active principle is known by other terms assigned by several investigators[1] proconvertin and convertin of Owren, co-thromboplastin of Mann, and Factor VII of Koller The latter two investigators in contrast to Alexander and colleagues and Owren do not accept the concept of conversion during coagulation, of a relatively inert plasma form of this substance into the active principle in serum

The following methods for assay of the Spca system are based upon the concept for which there is substantial evidence that activation of pro-Spca (proconvertin) into Spca (convertin) by thromboplastin and calcium does indeed occur and that the greater clot promoting activity of serum over and above that of the parent plasma is largely due to its content of active Spca

For the specific determination of the Spca system it is essential that all factors required in the prothrombin conversion mechanism except for the Spca system be provided in optimal amounts Since Spca acts via the thromboplastin Ca prothrombin conversion mechanism Spca can be measured only by its effect on the conversion of prothrombin to thrombin As mentioned above without it prothrombin conversion is extremely retarded the one-stage prothrombin time thus being markedly elevated The addition of Spca to plasma rendered deficient or devoid of Spca accelerates coagulation and shortens the prothrombin time It is therefore evident that present methods for the determination of Spca are only in

Successful utilization of this test requires strict control over the reagents both for the determinations of prothrombin and Ac globulin To be regarded as fully satisfactory they should give when used in testing human or bovine plasma (1) Bovine or human prothrombin values by the modified two-stage method of 250–300 units per ml (2) Plasma Ac globulin curves with good contour for normal bovine plasma diluted 3000–5000 times in the range 32–128 on the interpolation chart (3) Plasma Ac globulin curves with good contour, for human plasma diluted 1000 times, in the range of 12–48 on the chart

Multiple Analyses When the reagents are standardized several Ac globulin tests may be run at the same time By preparing the fibrinogen ahead of time it will be possible, by utilizing the latent period of each test (this is the time intervening after the mixing of the conversion mixture and the diluted plasma, before there is any detectable amount of thrombin formed), to set up new determinations at two and one half minute intervals If this is done five determinations can be done at the same time This will enable the investigator to run a control simultaneously with four plasmas However the number of points that can be plotted will be reduced due to the overlap of some of the dilutions but since this is a determination of rate and curves are used in the calculation of the answer, this will still be accurate for most of the work to be done With fewer tests closer intervals of time between determinations of clotting time can be done if greater accuracy is desired

Serum Ac globulin analysis Ac globulin when detectable in serum gives a different type of curve than that shown by plasma Ac globulin Conditions for testing for the serum Ac globulin are the same as those for the plasma type A special set of quantitative curves are, however, necessary for the final interpolation These are illustrated in figure 2 The difference in the curves given for the plasma type and the serum type Ac globulin is attributed to the fact that the former is activated along with thrombin formation causing an initially greater delay in thrombin appearance but more rapid prothrombin conversion when finally under way whereas the latter is already active when the conversion of prothrombin is begun

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Arnold G Ware and Walter H Seegers Serum Ac globulin Role in blood coagulation partial purification properties and quantitative determinations Am J of Physiol *152* 567 1948

Arnold G Ware and Walter H Seegers Plasma Ac globulin Partial purification quantitative determination and properties J Biol Chem *172* 699 1948

Arnold G Ware and Walter H Seegers Two stage procedure for the quantitative determination of prothrombin and Ac Globulin Am J Clin Path *19* 471, 1949

exhibits between 40–270 per cent enhancement of the prothrombic activity of plasma to which the serum is added with a mean of about 100 per cent[7] The standard deviation of a series of observations on a single serum sample which ranged from 138 to 168 per cent enhancement with a mean of 149, was ±8 6

Precautions and Sources of Error Many of the conditions which influence the serum Spca activity have been studied[7] Since it is known that the addition of thromboplastin to freshly hed blood undue agitation or excessive foreign surface exposure of blood will increase the Spca activity in the serum certain precautions in the sampling of the blood deserve emphasis Glassware and needles must be scrupulously clean venipuncture should be direct the blood should flow freely with no frothing and undue agitation of the blood should be avoided The precise relation of volume of blood to the glass surface to which it is exposed should be defined in studies of the dynamics of Spca formation under various conditions

Additional precautions in actual testing as well as in interpretation of the results derive from recent knowledge regarding the Spca system In contrast to its inert plasma precursor (pro-Spca) Spca is relatively labile in serum and deteriorates while at the same time some conversion of pro-Spca into its active form continues slowly Thus at any given time the amount of Spca activity that is manifest is the resultant of two forces—destruction of the active Spca and elaboration of Spca from its precursor The latter requiring calcium is greatly retarded by decalcification of the serum Since only 10–20 per cent of the pro-Spca is converted to the active form during spontaneous coagulation the Spca system in decalcified aged serum consists preponderantly of the more stable pro-Spca The implications of these facts with regard to the most opportune time for oxalating the serum for performing the test etc are clear

Certain limitations of the procedure also become evident after careful scrutiny of the conditions under which the test is performed The $BaSO_4$ adsorbed normal human plasma used as diluent is devoid of both prothrombin and pro-Spca In order to test for Spca prothrombin must be provided This can be done only by adding whole normal plasma in a ratio of 1 to 20 of diluent which contributes to the total mixture about 5 per cent of normal prothrombin and 5 per cent pro-Spca * Under certain pathologic conditions where prothrombin consumption is poor and residual

It would be preferable to add optimal amounts of purified human prothrombin devoid of Spca Since such preparations have not yet been attained we must now use small amounts of plasma In so doing the total system is hypoprothrombinemic a condition contrary to the original statement that *all* coagulation factors concerned with prothrombin conversion (including prothrombin) except for the Spca system should be provided in optimal amounts Moreover the pro Spca which is also added exerts its effect by being converted to Spca during the test

direct and must be based upon the assay of prothrombin activity in systems critically deficient in Spca but otherwise intact in those other components involved in prothrombin conversion and in the thrombin fibrinogen inter action These involve the one-stage prothrombin procedures

Two methods for measuring Spca are described (a) The procedure of de Vries et al[8], (b) the method of Owren [9]

Determination of Spca by the Method of de Vries et al

Reagents and Apparatus These are the same as those employed in the one stage determination of over all plasma prothrombic activity, described on page 90

Performance of the Test 0 05 ml of a pool of normal oxalated plasma are mixed with 0 9 ml of pooled normal human $BaSO_4$ adsorbed plasma and 0 05 ml of physiologic saline solution, and the prothrombin time is determined on 0 10 ml of the mixture The prothrombic activity, calculated by interpolation on the standardization curve (obtained by using human $BaSO_4$ plasma as diluent), will usually be 5 per cent of normal, or 100 per cent when corrected for the total dilution

The same test is done on a mixture comprising 0 05 ml of the whole plasma, 0 9 ml of $BaSO_4$ plasma and *0 05 ml of oxalated serum* (or test material containing Spca) The prothrombic activity of this mixture is calculated from the prothrombin time by interpolation on the standardization curve, and similarly corrected for the degree of dilution of the whole plasma in the total mixture

The prothrombic activity of the serum alone is also determined in the same manner Since the activity of serum is usually low, the proportion of serum to $BaSO_4$ normal plasma in the mixture to be tested should be 3 to 7 Such proportions assure adequate amounts of nonprothrombin factors (other than Spca) which affect the prothrombin time

Values and Calculations In the calculation of serum Spca the algebraic sum of the individual prothrombic activities of the plasma and serum are subtracted from the observed prothrombic activity of the plasma serum mixture For example Assume the plasma to contain 100 per cent (of normal) prothrombic activity, and the serum 10 per cent (as computed from the standardization curve) A mixture of equal volumes of both should show a total of 110 per cent activity after suitable correction for dilution If the observed value is 240 per cent prothrombic activity, the enhancement of the prothrombic activity caused by the Spca is

$$\frac{240 - 110}{110} \times 100 = 118\% \text{ enhancement}$$

Normally serum obtained within several hours after coagulation

material is added to 0 1 ml of the bovine filtered plasma in a prothrombin time tube the mixture is allowed to come to bath temperature (37 5°C), 0 2 ml of the calcium thromboplastin reagent (maintained at 37 5°C) are added, and the prothrombin time is measured

Values and Calculations All determinations are expressed in terms of the activity of normal plasma * A standardization curve for the particular bovine plasma and thromboplastin used, is derived from the observed effects of normal pooled (from at least five subjects) human oxalated plasma upon the prothrombin time of the prothrombin rich bovine plasma rendered devoid of the Spca system by Seitz filtration The effect of a 1 to 10 dilution of the normal plasma with VBOS is taken as the 100 per cent standard activity further subdilutions of this giving correspondingly lower values The observed prothrombin times are plotted against the per cent activity (fig 1)

The proconvertin convertin activity of a test material also appropriately diluted with VBOS is computed by interpolating the observed prothrombin time on the standardization curve

Individual plasmas vary in activity from approximately 70–120 per cent of that of pooled plasma from a large number of normal subjects

Limitation It should be noted that the principle underlying this test is analogous to that for measuring prothrombin by the one-stage procedure where difficulty may arise in distinguishing between small amounts of pre formed thrombin contaminating the prothrombin Whereas in this instance preformed thrombin may be detected by direct clotting of the oxalated plasma or fibrinogen before the addition of thromboplastin and calcium the analogous distinction between proconvertin and convertin cannot be made in the proconvertin convertin method described

Precautions and Sources of Error Accordingly precautions and sources of error described above in the drawing of the blood sample and the preparation of serum are equally pertinent to this method Normal serum containing an appreciable fraction (approximately 10–20 per cent) of the convertin system in the activated form exhibits considerably greater activity than the corresponding plasma from which it was derived Thus anything which favors proconvertin activation (contamination of needle and syringe with tissue juice from inept venipuncture from agitation of the blood from excessive air blood interface etc) will give higher values

* At first glance this may appear as a contradiction in terms in view of what has been said about the relative inertness of the pro Spca in plasma in contrast to the active form Spca This is not so inconsistent when one realizes that the thromboplastin and calcium convert the pro Spca to Spca which then acts on the thromboplastin calcium prothrombin and Ac globulin in the system The profound clot promoting effect of minute amounts of whole normal plasma is evident from a typical standardization curve (fig 1)

serum prothrombin is high (e g hemophilia, thrombocytopenia) the conditions for testing the serum Spca are different, in that the test system will now contain a far greater concentration of prothrombin (provided by the serum) *in relation to the amount of Spca available Obviously until more* is known regarding the quantitative interrelationships between prothrombin conversion and Spca concentration, data obtained by the above method must be interpreted with caution In this connection the method of Owren has certain advantages

Determination of Proconvertin Convertin by the Method of Owren

General Considerations and Principle The procedure is essentially a one stage prothrombin method employing the apparatus and many of the reagents already described The test mixture comprises bovine oxalated plasma rendered devoid of the Spca system by filtration through a Seitz pad consisting of 20 per cent asbestos Such a plasma rich in Ac globulin and fibrinogen also contains 60 per cent of its original prothrombin, yet has a markedly retarded prothrombin conversion

Reagents and Apparatus

(a) Seitz filtered bovine oxalated plasma Freshly shed bovine blood obtained at the slaughterhouse is collected in 0 15 M sodium oxalate as described on page 94 The plasma is then filtered slowly under positive pressure through a 20 per cent asbestos Seitz pad * *Only that portion of* the plasma with a prothrombin concentration of 60 per cent of normal and a prothrombin time exceeding 100 seconds is used The filtered plasma is subdivided into aliquots each of sufficient volume for one day s supply These are kept in the frozen state (−15 to −20 C) under such conditions *the material is stable for at least 6 months*

(b) Calcium thromboplastin extract reagent as described for the specific determination of prothrombin

(c) Veronal buffered oxalated saline (VBOS) as described in the prothrombin procedure (page 94)

Performance of the Test The test material (serum serum fractions or plasma fractions) are suitably diluted† with VBOS 0 1 ml of the diluted

* Obtained from Carlson Ltd London England The speed of filtration and the vol filtered in relation to the size of the pad are important variables determined by trial and error in obtaining the desired result In general a 6 cm pad should be used to filter a vol of 100 ml of plasma at a rate of 40-60 drops per min The first 10-15 ml usually devoid of both prothrombin and pro Spca are discarded The next portion (about 50-60 ml) is usually rich in prothrombin but devoid of pro Spca A 14 cm pad can handle approximately 275 ml

† At least tenfold

Assay of Total Potential Convertin by a Two-Stage Modification of the Owren Procedure

General Considerations and Principle It would again be convenient to consider the proconvertin convertin system analogous to the prothrombin thrombin system Just as prothrombin can be measured only after it has been converted to thrombin, so proconvertin can be measured only after its conversion to convertin The determination of total potential convertin is carried out in two stages (1) incubation of the material with thromboplastin and calcium in order to convert the pro-Spca to Spca and (2) assay of the evolved Spca in the Spca free Seitz filtered bovine plasma rich in prothrombin

Reagents and Apparatus These are the same as those described above for the one stage proconvertin convertin determination by the Owren technic

Performance of the Test One volume of test material suitably diluted with VBOS* is added to two volumes of the $CaCl_2$-thromboplastin mixture and the combination is incubated at 37 5°C At intervals (about every three to 5 minutes) a 0 3 ml aliquot of the mixture is taken out and added to 0 1 ml of the filtered bovine plasma (at bath temp), and the clotting time is determined The observed clotting times become shorter as more and more of the proconvertin becomes activated The shortest clotting time (usually obtained with an aliquot after 15–20 minutes' incubation of the mixture) is taken as the point representing maximal convertin activity With further incubation the activity falls off due to destruction of the convertin especially in serum presumably caused by factors in serum (or plasma) which destroy it [8] To those who are thoroughly familiar with the two-stage plasma prothrombin method where the maximal thrombin titer evolved is measured before antithrombin exerts its effect this procedure for total convertin will appear reasonably uncomplicated

The per cent activity calculated by interpolation on a standardization curve derived as described above (example in fig 1) is expressed in terms of the activity of plasma For example the activity of 0 1 ml of a 1 10 dilution of normal human plasma with VBOS, added to 0 1 ml of bovine Seitz filtered plasma plus 0 2 ml of $CaCl_2$ thromboplastin solution is arbitrarily taken as 100 per cent activity Under the same conditions serum from spontaneously clotted blood will exhibit more activity (approximately 130 per cent) than the parent plasma After prior incubation of the serum with thromboplastin calcium the activity may reach values of approximately 500–1300 per cent of normal plasma due to complete con

* Since normal serum and Spca rich plasma or serum fractions can frequently be activated from 5 to 10 fold a suitable dilution with VBOS is generally 1 20–1 50

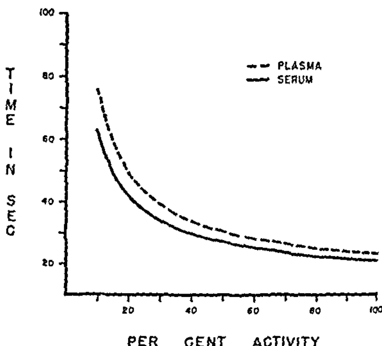


FIG 1—Typical standardization curve correlating prothrombin time with proconvertin convertin activity Pooled normal human oxalated plasma (or serum) added in various proportions to bovine plasma filtered through 20 per cent asbestos Seitz pad 100 per cent activity corresponds to 0 10 ml of a 1 10 dilution of normal plasma with VBOS added to 0 1 ml of the bovine plasma To this are added 0 2 ml of the thromboplastin calcium reagent

Note the relatively greater activity of equivalent amounts of serum compared with plasma

It is also important that the prothrombin content of the bovine filtered plasma be at least 60 per cent of normal Under such conditions the addition of prothrombin in the test material (plasma serum or fractions) which has been suitably diluted with VBOS prior to addition introduces a negligible increase in the prothrombin concentration of the total system If the bovine plasma reagent gives a prothrombin time in excess of 100 seconds and yet contains 60+ per cent prothrombin one may be assured that the reagent is virtually devoid of the Spca system

It is evident that with the above test it is impossible to estimate what fraction of the Spca system in a given test material is convertin or proconvertin since both will correct the retarded prothrombin conversion of the clotting mixture Additional information in this regard can be obtained by employing the procedure delineated below

4 Method of Assay of Cothromboplastin in Plasma or Serum

F D MANN

Object of Assay To determine specifically the blood coagulation factor cothromboplastin

Principle Thromboplastin reacts with cothromboplastin before it reacts with prothrombin This cothromboplastin reaction is slower than the other reactions involved in the conversion of prothrombin to thrombin and hence is responsible for a preliminary lag in the conversion of prothrombin to thrombin If thromboplastin is treated with very dilute plasma for three minutes very little formation of thrombin occurs but when subsequently a higher concentration of plasma is added the preliminary lag in the formation of thrombin is partially eliminated Serum and preparations of cothromboplastin are assayed in the same way

Reagents (1) Physiologic saline solution 0 9 per cent NaCl (2) Calcium chloride saline solution 100 mg anhydrous $CaCl_2$ dissolved in 200 ml of 0 9 per cent saline solution (3) Imidazole buffer pH 7 2 0 86 Gm imidazole dissolved in 46 ml 0 1 normal HCl and made up to 50 ml volume with H_2O (4) Thromboplastin 0 15 Gm of acetone dehydrated rabbit brain ground in a mortar with 15 ml calcium chloride saline solution and the mixture allowed to stand for 10 minutes with intermittent stirring then centrifuged for two minutes at 1000 rpm in a No 2 International centrifuge Supernate poured off and diluted with twice its volume of calcium chloride saline solution (5) Acacia Twelve Gm gum acacia dissolved in 100 ml of 1 1 per cent disodium acid phosphate (Na_2HPO_4 12 H_2O) Solution centrifuged and clear supernatant poured off (6) Fibrinogen 1 per cent bovine Fraction I (7) Fibrinogen acacia Equal parts of (5) and (6) mixed immediately before use (8) Normal human plasma From normal human blood mixed with 1/9 volume of M/10 sodium oxalate (9) Defibrinated plasma To (8) add 1/10 volume of thrombin (10 units/ml) after 15 minutes remove clot

Apparatus (1) Electric stop clock operated with foot pedal (2) Glass constant temperature bath at 28 C

Procedure (1) 1 0 ml of thromboplastin (including calcium) added to 0 5 ml of 1 300 dilution normal plasma in imidazole buffer (2) After exactly three minutes at room temperature 0 15 ml of mixture (1) added to 0 05 ml of a 1 25 dilution of defibrinated normal human plasma (3) After exactly one minute at room temperature 0 2 ml of fibrinogen acacia added to mixture (2) and the clotting time determined in a water bath at

version of proconvertin to convertin Theoretically, the difference between the activity of the unincubated and thromboplastin Ca incubated serum (or fractions) will reflect that amount of the Spca system originally in the proconvertin form

Precautions and Sources of Error It should be pointed out that unfortunately this procedure cannot be applied to materials (plasma or plasma fractions) which also contain prothrombin for, obviously, incubation with thromboplastin and calcium will evolve thrombin which will then rapidly clot the filtered bovine plasma

The degree to which destructive forces, acting on evolved convertin cause *erroneously low values cannot at present be surmised In our experience* purified human serum fractions, rich in the Spca system maintain their total convertin titer remarkably well (>30 minutes) after the incubation peak is reached in contrast to what is observed with whole serum

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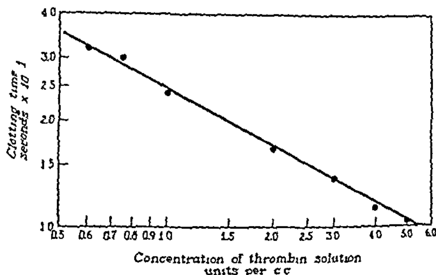


Fig 1 —Relationship between thrombin concentration and clotting time when 0 2 cc of fibrinogen acacia mixture was added to 0 2 cc of thrombin solution (From Hurn M, and Mann F D Amer J Clin Path *17* 741, 1947)

28°C (4) 0 5 ml of unknown solution made up in imidazole buffer tested exactly like the normal plasma in steps (1), (2) and (3)

Calculation (1) Thrombin unitage corresponding to clotting time in (3) above read from curve in figure 1 (2) Cothromboplastin activity of unknown calculated as follows

$$\frac{\text{One minute thrombin yield of unknown}}{\text{One minute thrombin yield of control}} \times \frac{\text{dilution of unknown}}{\text{dilution of control}} \times 100$$

$$= \% \text{ cothromboplastin activity}$$

Example One minute clotting time when normal plasma tested in 1 300 dilution = 25″ One minute thrombin yield = 0 95 units One minute clotting time when specimen of dicumarol plasma tested in 1 30 dilution = 28″ One minute thrombin yield = 0 75 units

$$\frac{0\,75}{0\,95} \times \frac{30}{300} \times 100 = 7\,9\% \text{ cothromboplastin activity}$$

Normal range of plasma cothromboplastin activity in man 60 to 140 %

Precautions and sources of error (1) For consistency plasma from the same normal individual used in a series of determinations (2) Some extracts of thromboplastin, although satisfactory for the two stage determination of prothrombin are insensitive indicators of cothromboplastin activity, these must not be used

aspiration and ejection from the syringe During this mixing process the plunger of the syringe itself may be removed and immersed into the diluted mixture once before the final fluxing this reduces the risk of excessive carry-over from one dilution to the next particularly during the first dilutions To each tube is added 10 units of thrombin (0 1 ml of the solution from a pipette or 1 drop from a dropper) Mix well Coagulation occurs in a few seconds and is complete in a few minutes

Readings The rack of tubes is allowed to stand at room temperature preferably without shaking until the time of reading Each tube is then tilted back and forth to find the highest dilution in which a visible coagulum has formed In the dilutions near the end point the fibrin may contract to a minute clot or, indeed if there has been shaking may accumulate directly as a minute clot

Controls Because of the infrequency with which one may use the fibrin titer assay, it is desirable to perform a control titer at the same time on the blood of one or more normal pregnant women near term or just delivered to provide a check on the technic When several such comparisons are made it is found that a difference of one tube is not necessarily significant

A control sample from a nonpregnant individual may be used if it is kept in mind that the fibrinogen content is approximately 250 mg per cent rather than the usual 500 mg per cent that is more characteristic of normal term pregnancy still higher levels may occur in toxemia of pregnancy

A titer of 800 is commonly found in normal pregnant women near term Variations in the titer assay are usually limited to one tube in either direction by observation of careful technic of dilution

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CHAPTER IX

FIBRIN AND PRECURSORS

1 Estimation of Plasma Fibrinogen (Rapid Method of Schneider)

Adapted by L M TOCANTINS

Principle Plasma is diluted and the highest dilution in which visible coagulation occurs when thrombin is added is termed the 'fibrin titer"

Reagents and Apparatus (a) Glass tubes 12 x 100 mm (b) Ringer's solution (or alternatively a solution made up of 9 parts 0 85 per cent NaCl and 1 part of 1/40 M CaCl) (c) Thrombin (bovine "topical thrombin') dissolve the contents of 1 vial in enough 50 per cent glycerol to make a solution of 200 units per ml This keeps well in the refrigerator for months

Steps in the Procedure

Arrange two rows of tubes, 8 in each row Label one "patient,' the other 'normal control"

Dilutions First place 3 ml of the diluent in the second tube of each row of tubes, 4 ml in the third tube and 1 ml in each of the remaining tubes

Venous blood is then drawn from the patient into a syringe of small diameter (a 1 ml or 1/2 ml tuberculin syringe or insulin syringe is of small enough diameter to be admitted deeply into the dilution tubes for mixing and sampling) and is dispensed and diluted, at once directly from the syringe, at the bedside into the series of assay tubes in the following manner An undiluted control sample is set aside by discharging all but 0 5 ml of the blood from the syringe into the first tube The remaining 0 5 ml of whole blood is then discharged into the second tube and is thoroughly mixed with the 3 ml of diluent already in it With the same syringe 1 ml of this mixture is carried over to the next tube The remaining twofold dilutions are accomplished by carrying over a 1 ml aliquot serially from each tube to the next Assuming a hematocrit of approximately 0.35, the resultant dilutions of the plasma component are 1 10 50 100, 200 400 800, 1600

With care the original syringe may be used to make the dilutions In each successive dilution, the mixing may be accomplished by repeated

Range of values In man the range of normal values estimated by a similar method[1] is 190 to 330 mg per 100 ml

REFERENCE

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3 Estimation of Fibrinogen In Small Samples of Plasma (Method of Ratnoff and Menzie)

Adapted by R R HOLBURN*

Principle The amount of fibrin precipitated from plasma and adhering to glass particles is determined colorimetrically by the addition of Folin Ciocalteu phenol reagent to alkaline solutions of the fibrin The optical density of the developed color is directly proportional to the concentration of fibrinogen or tyrosine present and may be measured by comparison with the color developed by a known amount of a tyrosine standard

Apparatus and Reagents (1) *Plasma* Venous blood is transferred immediately after withdrawal to a test tube containing 0 2 ml oxalate mixture for each 5 ml blood The oxalate mixture contains 0 8 mgm of potassium oxalate and 1 2 Gm ammonium oxalate per 100 ml solution The plasma is separated by centrifugation for 10 minutes at 2000 rpm in a Sorvall angle centrifuge (2) *Crushed glass* Pyrex glass is crushed in a mortar so that the largest diameter of the particles is approximately 0 5 mm The glass is not graded nor is the glass dust removed Between each use the glass particles are washed with chromic acid and then well rinsed and dried (3) *Thrombin* Commercial thrombin containing 1000 units per ml (4) *Sodium hydroxide* 10 grams per 100 ml distilled water (10 per cent) (5) *Sodium carbonate* 20 grams per 100 ml distilled water (20 per cent) (6) *Folin Ciocalteu phenol reagent* For preparation see J Biol Chem 73 627, 1927 (7) *Tyrosine standard* 200 mg tyrosine per liter of 0 1 N hydrochloric acid (8) *Coleman Junior Spectrophotometer*

Procedure To a 40 ml round bottom pyrex centrifuge tube is added approximately 0 5 ml ground glass 10 ml of 0 85 per cent NaCl solution and 0 05 ml of the thrombin solution Then 0 5 ml plasma is pipetted into the tube which is agitated with an oscillatory motion Volumes of plasma

From Journal of Laboratory and Clinical Medicine 37 316 1951

2 Estimation of Plasma Fibrinogen

L M TOCANTINS

Principle Citrated plasma is diluted, recalcified, the fibrin is separated, washed and its N content measured by the Kjeldahl method

Reagents 19 per cent trisodium citrate 0 85 per cent NaCl 0 2 M $CaCl_2$ Kjeldahl reagents

Steps in the Procedure

Collect 5 ml of blood into 0 1 ml 19 per cent trisodium citrate centrifuge, and obtain clear plasma Measure out two 0 5 ml portions and treat each alike as follows

(1) Place 0 5 ml of the plasma into a round bottomed tube measuring approximately 17 mm internal diameter and 11 1/2 cm length

(2) Add 9 5 ml of 0 85 per cent NaCl solution and 0 05 ml of 0.2 M $CaCl_2$ and mix well Place a rubber stopper in the tube and incubate for one hour at 37°C

(3) Remove clot by running a fine capillary tube or fine glass rod carefully between the clot and the wall of the tube dislodging the clot gently and wrapping it around the tube or rod This has to be done very gently to prevent breaking the clot

(4) Remove the clot from about the glass rod or tube by pushing it down with the fingers covered with filter paper Place the clot on N free filter paper Press it gently against the paper to squeeze out most of the plasma

(5) Prepare a glass funnel provided with a piece of rubber tube at its lower end and a spring clip

(6) Place a fresh piece of N free filter paper in the funnel and wash the fibrin clot with at least three changes of normal salt solution Allow each change to remain in the funnel for five to ten minutes before releasing the clip

(7) Place the fibrin clot in a Kjeldahl flask holding the digest mixture The acid digest is distilled and the distillate diluted nesslerized and compared with N standard In case the color of the unknown is too strong for comparison with the standard the unknown is diluted further and proper allowance is made for this dilution in the calculation of the results If the unknown is too weak the specimen is diluted less

(8) Transfer nitrogen values into protein values by multiplying by 6 25 Express results in terms of mg of fibrinogen per 100 ml of plasma

(9) The values of the two samples are averaged if the difference between them is less than 10 per cent of the mean

tivity and the nitrogen content of duplicate samples of fibrin (The accuracy of the factor may be checked by measuring the amount of fibrinogen in a large plasma sample gravimetrically) The calculated answer is multiplied by 1 08 to correct for the dilution by the oxalate and the results reported for 100 ml of plasma

Values Obtained In 19 normal men the fibrinogen content of the plasma varied from 193 to 415 mg per 100 ml averaging 272 mg per 100 ml of plasma In 14 normal women the fibrinogen content varied from 164 to 366 mg per 100 ml of plasma averaging 294 mg There is no statistical significant difference between the concentration of fibrinogen in men and women

Precautions and Sources of Error A small error may be introduced into the determination of plasma fibrinogen by the adsorption of other proteins on the fibrin clot

If glass is not used in the formation of the clot the time required for the full development of color is lengthened to 30 minutes

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4 Preparation of Fibrinogen

J F JOHNSON and W H SEEGERS

In analytic experiments it is essential that there be a constant source of active purified fibrinogen in order to insure accuracy and reproducible results Commercial preparations if such are available are usually not adequate containing only from 75 to 90 per cent clottable protein A simple method to obtain fibrinogen based upon the observation that fibrinogen is not completely soluble in cold plasma is here described This method carefully carried out regularly yields a product which after freezing and thawing remains clear for many hours and is free of prothrombin

as small as 0 1 ml can be used As clotting occurs the fibrin adheres to the glass particles With active preparations of thrombin, clotting is complete in 2–3 minutes If clotting continues after the cessation of the shaking, further agitation and rotation results in adherence of the new clot to the glass After ten minutes the tube is centrifuged for 5 minutes at 2000 rpm in the angle centrifuge The supernatant is discarded The clot is washed twice by adding 10 ml of the 0 85 per cent NaCl solution to the tube and expressing it carefully against the wall with a glass rod The fibrin is separated by centrifuging for three minutes and decanting the supernatant One ml of 10 per cent NaOH solution is then added to the tube and the mixture boiled for 10 minutes Evaporation is minimized by covering the water bath

After the tube cools, 7 ml water is added, followed by 3 ml of the sodium carbonate solution and 1 ml of the Folin Ciocalteu reagent The blue color is fully developed in 10 minutes If crushed glass is not used thirty minutes is required A 1 ml aliquot is diluted with 3 ml water The intensity of the color is read in the spectrophotometer at a wave length of 650 mμ

A blank is made up in the same fashion except for the addition of plasma One ml of the standard tyrosine solution is pipetted into a 40 ml centrifuge tube and in succession, are added 1 0 ml sodium hydroxide solution 6 0 ml water 3 0 ml sodium carbonate and 1 0 ml of the phenol reagent One ml of the solution is diluted with 3 0 ml water and the intensity of the blue color read after ten minutes against a blank This blank is not the same as that prepared for reading the color of the fibrin solution The blank is prepared in the same manner as the tyrosine standard, except that 1 0 ml of water is substituted for the tyrosine

Calculation The optical density of the developed color is directly proportional to the concentration of fibrinogen or tyrosine until an amount equal to 0 35 mg of tyrosine is reached When the intensity of color in an unknown solution is greater than that value, the equivalent amount of tyrosine is estimated from a calibration curve

Calibration curve Varying amounts of tyrosine are diluted with water to a volume of 7 0 ml Then 1 0 ml sodium hydroxide solution, 3 0 ml sodium carbonate solution 1 0 ml phenol reagent are added After 10 minutes 10 0 ml is diluted with 3 0 ml water and the intensity of color read against a blank

The amount of fibrinogen in the plasma sample is calculated by reading from the calibration curve the weight of tyrosine giving an equivalent amount of color The weight of tyrosine is then multiplied by the factor necessary to express the value per 1 0 ml of plasma The last result is multiplied by 11 7 the conversion factor measuring the tyrosine like ac

ing the material is returned to the cold centrifuge cups and centrifuged again at 1250 g for about 1 minute After this centrifugation the saline supernatant is discarded the fibrinogen is removed and the mixing with saline is repeated The amount of saline used each time is gradually de creased The washing is repeated at least five times and more times may be required before a final white fibrinogen is obtained The last washing should be for three minutes, at the former speed to remove most of the saline

The material is then suspended in about 200 to 300 ml of saline and placed in a water bath at 35° C The hand stirrer is again used to avoid denaturation and to insure complete heating of the mixture by keeping the liquid in constant motion until all the fibrinogen is dissolved and the final temperature is about 33 C

The solution is then placed in warm centrifuge tubes and centrifuged at room temperature for 2 hours at 1250 g Following this the clear opal escent solution of fibrinogen is decanted into a graduate and the volume measured A 2 or 3 ml sample is taken for the analysis to be described

The product itself is frozen with the aid of dry ice and alcohol and stored in the deep freeze for many months or it can be freeze dried and stored in the powdered form for future use When such frozen products are thawed they are placed in a hot water bath If the hands can be immersed in the hot water comfortably the fibrinogen container can be rotated until all ice is gone Slow thawing without motion denatures purified fibrinogen

Should the material be needed for analytical use at once it is made up into a one per cent solution by dilution with 0 85 per cent NaCl solution This final solution should contain 10 per cent imidazole buffer by volume For storage it is placed in small serological tubes 10 by 75 mm and quick frozen To facilitate freezing it is convenient to make an aluminum block in which holes are drilled of such size that 12 x 75 mm serologic tubes will just fit into the holes This block is then placed in a freezing mixture of alcohol and dry ice The small tubes are put in the holes and fibrinogen is pipetted into them The solution freezes almost at once After this proc ess the tubes are placed in the deep freeze until they are thawed for use

After the whole procedure is finished the containers and instruments used can be cleaned with a strong solution of sodium hydroxide They can be set to soak in this mixture and on returning the next day they are easily cleaned Needless to say all these instruments and containers should be scrupulously clean before use The usual procedure is to use chromic and sulfuric acid cleaning solution on all apparatus This should be removed thoroughly with water and saline rinses before use

Estimation of Clottable Protein in the Fibrinogen Preparation One ml of fibrinogen solution to be tested is mixed with 30 ml of 0 85 per cent

fibrinolytic enzymes, and other elements concerned in the clotting reaction It remains stable and maintains its reactivity for many months in the deep freeze

Materials and Reagents (a) Plasma The blood used should be collected in a special anticoagulant (1 85 per cent $K_2C_2O_4$ $2H_2O$ and 0 5 per cent $H_2C_2O_4$ $2H_2O$), because the resulting plasma has a low salt concentration The usual procedure is to collect the blood at the slaughter house The plasma is separated from the blood by centrifugation This plasma is then frozen solid at deep freeze temperatures

(b) Saline 0 85 per cent NaCl is used throughout This has either been pre cooled to 0°C or has been placed in the cold overnight where it may even be frozen and then used as it thaws A suitable buffer for use in the dilution of fibrinogen is prepared by dissolving 1 72 grams imidazole in 90 ml of 0 1 N HCl and then diluting with distilled water to 100 ml The final solution will be of a pH of 7 2 to 7 4 If this is not achieved the end point can be adjusted with a few drops of NaOH or HCl

Steps in Procedure About four gallons of the frozen plasma is broken into small chunks and force-thawed in a metal container An electric fan directed at the container will aid in the supply of some energy by convection It is important not to over heat the plasma It should be examined frequently and stirred to insure uniform thawing When the mass has become a slush with only about 1/20 of the total remaining as ice it is ready for centrifugation By close examination of the plasma, the fibrinogen can be seen as particulate material throughout the fluid

This material is then centrifuged in glass centrifuge tubes or chilled brass trunnion cups (International Equipment Co 2½" i d by 4½" deep) at room temperature at 1250 g for one minute The supernatant is poured off and a thin layer of fibrinogen can be seen lining the bottom of the cups This layer is allowed to remain and with a new supply of plasma in the same cups the centrifugation is repeated until all the fibrinogen has been harvested

It is emphasized that the preservation of low temperature is essential in all phases of this procedure The brass trunnion cups or centrifuge tubes should be returned to the ice bath when not being handled and all objects used should be cool If a cold room is available that should be most nearly ideal

Following this initial spinning the fibrinogen is removed from the cups and deposited in a large metal beaker that has been placed in an ice bath An equal volume of ice cold saline is poured over the mass and the whole mixture is stirred by a hand stirrer The device used is made by placing a No 7 rubber stopper on the end of a strong glass rod It is used as a stomper Mechanical stirring denatures the fibrinogen After thorough mix

CHAPTER X

FIBRINOLYSIN PRECURSORS AND INHIBITORS

1 Estimation of Fibrinolytic Activity (Fibrin Plate Method of Astrup and Mullertz)

Adapted by R R HOLBURN*

Object of Method A simple and convenient method for the determination of fibrinolytic activity giving an accurate assay with a wide variety of materials

Principle Fibrinolytic enzymes will lyse a standard fibrin clot leaving a clear area in an otherwise opaque plate of clotted fibrinogen The extent of the digested zone gives a measure of the fibrinolytic activity of the solution tested

Apparatus and Reagents (1) Petri dishes 10 cm diameter with uniform and level surfaces Care must be taken that the surfaces are parallel since the dishes are placed one on top of the other and any unevenness will result in unequal distribution of the fluid and the areas of lysis will not be circular The dishes are heat sterilized to avoid bacterial contamination of the fibrin during incubation (2) Fibrinogen The purest fibrinogen available is used and diluted with 0 85 per cent NaCl to a concentration of 0.2 or 0 4 per cent A fibrinogen preparation of 80 to 90 per cent clottable protein was used (3) Buffer To 662 ml of 0 1 M Sodium diethyl barbiturate is added 338 ml of 0 1 M HCl and then diluted with 320 ml water (resulting solution pH 7 8 ionic strength = 0 05) (4) Thrombin Purified thrombin in a final concentration of 100 units per ml in 0 85 per cent NaCl solution (5) Incubator Temperature of 37 C (6) Pipets 5 ml and 0 1 ml graduated in 0 001 ml

Procedure In each Petri dish is placed 9 0 ml of the diluted fibrinogen solution using the buffer as diluent (fibrinogen concentration 0 2 or 0 1 per cent) The Petri dishes are then put on a horizontal surface and clotted by the addition of 0 2 ml thrombin solution A clot is allowed to form and should be as even and homogeneous as possible Exactly 0 03 ml of the solution to be tested is added in the form of a drop onto the clotted surface

From Arch Biochemistry and Biophysics *40* 346-351 195?

NaCl solution containing 1 ml of M/5 phosphate buffer at pH 6 4, and 10 5 ml of 1 per cent $CaCl_2$ The calcium is not precipitated by the phosphate For convenience a 30 x 120 mm tube of 50 ml capacity is used The $CaCl_2$ produces a less soluble fibrin and fibrin formed at pH 6 4 is more easily rolled out than that formed in a more alkaline medium

To this diluted fibrinogen, 1 ml of a 50 per cent glycerol solution containing about 100 Iowa units of purified thrombin is added 'Thrombin Topical' may be used Clotting occurs in a few moments, the opalescent fluid becoming cloudy This mixture is allowed to stand for 30 minutes, either at room temperature or in the refrigerator

After the formation of fibrin the clot is rolled out with use of a stainless steel wire or rod, squeezing as much liquid from the clot as possible To facilitate this latter step the clot is removed from the solution and rolled out on filter paper with gentle pressure It is then washed twice with water and then twice with 0 85 per cent NaCl solution before being analyzed

The clot may be dried and weighed It may be analyzed for nitrogen or tyrosine In the latter case the clot is placed in 0 5 ml of 10 per cent NaOH for digestion The amount of tyrosine in the digested clot is then determined by the method of Folin Ciocalteu To find the purity of the product the tyrosine equivalent of the solution is found for a 0 5 ml specimen taken before the clot is formed with thrombin A comparison of the two values then in hand, gives the percentage of the total protein that is clottable fibrinogen

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2 Estimation of Fibrinolysin Activity of Human Plasma or Serum

E C LOOMIS

Object of the Test To determine the relative amount of profibrinolysin present in human plasma or serum

Principle Underlying the Test Human profibrinolysin is activated to fibrinolysin a fibrinolytic enzyme by streptokinase an exotoxin produced by certain strains of hemolytic streptococci The activated fibrinolysin will digest or lyse a fibrin clot

Reagents and Apparatus Required (1) Human plasma or serum to be assayed (2) Streptokinase 100 000 u /ml solution—(Lederle Laboratories Division American Cyanamid Co) (3) Bovine Fibrinogen 0 6 per cent clottable in imidazole buffered saline solution (Most lots supplied by Armour and Co suitable but the fibrinogen prepared by method of Ware et al [1] is preferred) (4) Bovine Thrombin (Parke Davis and Co) 75 u /ml in 50 per cent glycerol (5) Open ended capillary tubes (e g melting point tubes) (6) 10 x 75 mm test tubes (7) Pipets (8) Water bath at 45 C (9) Stopwatch

Steps in Performance of the Test If plasma is to be assayed convert to serum (remove fibrinogen) by recalcification or addition of thrombin Express the serum from the fibrin clot and assay If any dilution is made correct for it in the calculation below

Into a 10 x 75 mm test tube place 0 1 ml streptokinase 0 2 ml thrombin 0.2 ml of serum or a suitable dilution Add 0 5 ml of 0 6 per cent bovine fibrinogen rapidly and simultaneously start a stopwatch Quickly tilt or shake the test tube to aid mixing of the reagents As soon as a clot forms insert a capillary tube to the bottom of the clot and place in the water bath at 45 C The end point is taken at the instant the liquid level in the capillary rises to the clot level in the test tube

Calculation By definition a unit of fibrinolysin is that quantity which will lyse a 0 3 per cent fibrin clot in 2 minutes at 45 C in an imidazole buffered system at pH 7.2 This determination besides measuring fibrinolysin also includes a partial measurement of the rate of activation

$$\text{Units of Fibrinolysin/ml} = 5 \times CF \times D$$

Where D = dilution CF = correction factor for deviation from 120 seconds in the lysis time

Normal Range of Values This has not been established by this procedure since a very limited number of human assays have been done

Usually three single determinations are carried out on a plate The plates are then incubated 18 to 20 hours at 37° C and the product of two perpendicular diameters (square mm) of the digested area is calculated If especially accurate estimations are required, zones which are not circular are disregarded The areas are converted into concentrations by interpolation on a reference curve

Calculation For a number of enzymes a straight line is obtained when the logarithm of the product of the area is plotted against the logarithm of the concentration expressed in terms of a stock solution This relation is expressed by the equation

$$A = k \times c^a \quad \text{or} \quad \log A = a \log c + b$$

where A = area or product of two perpendicular diameters c is the relative enzyme concentration, a is a constant denoting the slope of the curve, and k and b are constants It is possible to compare two solutions only when the curves have identical slopes solutions differing in concentrations produce parallel dilution curves in a log log graph By means of such curves the concentration of the unknown solutions can be estimated by interpolation on a dilution curve of a solution used as a standard of reference

Values Obtained Bovine plasmin range a from 0 86 to 1 04, mean 0 97 Human streptokinase activated plasma (s plasmin) range of values of a from 0 46 to 0 60 mean 0 50 Trypsin inconsistent results with a 0.2 per cent fibrinogen solution, with a 0 1 per cent solution values of a ranging from 0 75 to 1 00, mean of 0 90

Precautions and Sources of Error (1) Deviation of the slopes of the curves (2) Variations in the positions of the curves above the abscissa

Discussion In estimating the reproducibility and the sensitivity of the method crystalline trypsin may be used as a standard of reference As small an amount as 0 02 μg can be detected The method is especially adapted for plasmin (fibrinolysin) but may be used as a practical tool in the study of other fibrinolytic enzymes

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3 Preparation of Fibrinolysin (Plasmin, Serum Tryptase)

E C LOOMIS

Early preparations of fibrinolysin were found to clot fibrinogen This has been attributed to the enzyme itself but it has been shown that clotting properties either direct or indirect through activation of prothrombin cannot be ascribed to fibrinolysin In order to prepare prothrombin and thrombin free fibrinolysin the prothrombin is removed from bovine and human plasma by adsorption on an excess of $Mg(OH)_2$ cream and by centrifugation to remove the adsorbate The profibrinolysin is then concentrated by fractionation and precipitation technics and activated

An alternate procedure which gives a far superior fibrinolysin product is the fractionation of profibrinolysin from serum by essentially the same method

Preparation of Serum Serum is prepared from oxalated bovine or human plasma by the addition of 0.3 per cent excess calcium chloride to the vigorously agitated plasma The fibrin precipitates in strands Stirring is continued for 30 minutes after the fibrin separates to allow for complete conversion of prothrombin to thrombin and for antithrombin (present in the serum) to destroy the thrombin

Each lot of serum is tested and should respond as follows (1) One ml serum + 0 1 ml 100 U thrombin → no clot Therefore all fibrinogen was removed (2) Two tenth ml serum + 1 ml purified fibrinogen → no clot Therefore all thrombin destroyed (3) One ml serum + 1 ml purified fibrinogen + 0 2 ml purified lung extract → no clot Therefore all prothrombin was converted to thrombin and destroyed as proved in (2)

Preparation of Plasma Four liters of plasma are treated with 600 ml of $Mg(OH)_2$ cream The $Mg(OH)_2$ with the adsorbed prothrombin is removed from the supernatant plasma by centrifugation for 10 minutes at 5 000 rpm

Separation of Fibrinolysin from Plasma or Serum The plasma (or serum) is cooled to 5 C and a saturated solution of $(NH_4)_2SO_4$ added dropwise with constant stirring to 25 per cent of saturation The precipitated proteins are removed by centrifugation for 3 minutes at 5 000 rpm The solids are discarded The supernatant solution is cooled to 0 C and the degree of $(NH_4)_2SO_4$ saturation increased to 29 per cent by the further dropwise addition of saturated $(NH_4)_2SO_4$ with constant stirring The precipitate is collected by centrifugation at 5 000 rpm for 3 minutes The supernatant solution is discarded

Correction Factor Table

Lysis time in seconds	CF	U ts/ml (5 × CF)	Lysis time in seconds	CF	Units ml (5 × CF)
90	1 32	6 60	120	1 00	5 00
95	1 25	6 25	125	0 96	4 80
100	1 20	6 00	130	0 94	4 70
105	1 13	5 65	135	0 92	4 60
110	1 10	5 50	140	0 88	4 40
115	1 05	5 25	145	0 85	4 25

Precautions and sources of error (1) Fibrinogen contamination with fibrinolysin As specified above bovine fibrinogen is the most suitable substrate reagent for the assay If the bovine preparation of Ware et al[1] is used, least difficulty will be encountered All human fibrinogen preparations used have been found to be contaminated with fibrinolysin to a much greater degree If human fibrinogen must be used, a 0 15 per cent clot instead of the 0 3 per cent bovine clot will give a unit approximately equal to the defined unit[2]

(2) Thrombin contamination with fibrinolysin or profibrinolysin Some investigators have found slight contamination of certain lots of Thrombin Topical (bovine) However since bovine profibrinolysin activates very poorly with Streptokinase the only error to be considered is the presence of the active proteolytic enzyme

(3) Streptokinase This procedure has been established using the Lederle preparation, which is not sufficiently purified for the ideal assay method[4] It very often contains an inhibitor of fibrinolysin

(4) Antistreptokinase and antifibrinolysin are present in the serum and cannot be removed or inactivated without harm to the profibrinolysin.

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4 Estimation of Antifibrinolysin in Plasma or Serum

E C LOOMIS

Object of the Test To determine the number of units of antifibrinolysin present in plasma or serum

Principle of the Test Serum is added to an excess of fibrinolysin and allowed to react for a standard time The excess fibrinolysin is then determined and the amount of antifibrinolysin is calculated by difference

Reagents and apparatus required (1) Plasma or serum to be assayed (2) Fibrinolysin (Parke Davis and Co) (3) Bovine Fibrinogen, 0 6 per cent clottable in imidazole buffered solution (most Armour and Co lots suitable but preferably prepared by method of Ware et al [1]) (4) Bovine Thrombin (Parke Davis and Co) 50 u/ml in 50 per cent glycerol (5) Open ended capillary tubes (e g, melting point tubes) (6) 10 x 75 mm test tubes (7) Pipets (8) Water bath at 45 C (9) Stopwatch

Steps in performance of test (1) If plasma is to be assayed convert it to serum (remove fibrinogen) by recalcification or addition of thrombin Express the serum from the clotted fibrin and assay Any dilution in this step should be corrected for in the calculations below

(2) Into a 10 x 75 mm test tube place at least 30 units of fibrinolysin dissolved in 0 9 ml physiologic saline buffered with imidazole (see page 165) Add 0 1 ml serum to be assayed Allow to stand for 60 minutes at 26–28 C then assay the remaining fibrinolysin

(3) Place 0 3 ml of 50 unit thrombin into a 10 x 75 mm test tube, add 0.2 ml of the solution from (2) (or suitable dilution of that solution) Start a stopwatch upon the addition of 0 5 ml of 0 6 per cent bovine fibrinogen As soon as a clot forms place the test tube in a 45 C water bath insert an open end capillary tube to the base of the clot The end point of the determination is reached when the liquid level in the capillary rises to the clot level in the test tube This should be between 90 and 145 seconds or a different dilution should be assayed The fibrinolysin solution used in (2) should also be assayed following the step being described

Calculations

$$A = 10\,(0.9\,F_O - F_R)$$

$$F = 5\,CF \times D$$

Where A = units of antifibrinolysin per ml of plasma or serum F_O = units of Fibrinolysin per ml of original solution F_R = units of Fibrinolysin per ml after 1 hour reaction C.F = correction factor for deviation from

The precipitate is dissolved in 100 ml distilled water, transferred to a separatory funnel and shaken intermittently for 30 minutes with 25 ml $CHCl_3$. After this activation, the $CHCl_3$ layer is separated and discarded. The aqueous phase is dialyzed for 16 hours against cold running tap water in Visking "No Jax" casings. The precipitate which formed on dialysis is collected by a short fast centrifugation, as above, dissolved in 100 ml. of physiologic saline, diluted to 1 500 ml with cold distilled water (to a specific resistance of the solution—approximately 400 ohms), cooled to 0°C and adjusted to pH 5 5 (glass electrode) with N HCl. The precipitate which forms is collected by a short, fast centrifugation, dissolved in 100 ml physiological saline, adjusted to pH 7 0 to 7 2 with 0 1 N NaOH, shell frozen and lyophilized.

Unit The unit of activity for fibrinolysin has already been defined as follows. One unit of fibrinolysin is that amount which will dissolve 1 ml of a 0 3 per cent fibrin clot in 120 seconds at pH 7 2 and 45°C in an isotonic saline system buffered with imidazole.

Assay Two-tenths ml of a saline solution of fibrinolysin of known concentration is added to 0 1 ml of 100 unit/ml thrombin solution in 50 per cent glycerol in a 10 x 75 mm test tube. Three tenths ml of a 0 6% fibrinogen solution containing imidazole buffer is then blown into the tube as a stopwatch is simultaneously started. The tube is placed in a water bath at 45°C, removed every 15 seconds and tilted gently. The endpoint of the assay is considered to be the earliest time the solution flows on gentle tilting of the tube.

Average of 6 preparations from beef plasma 0 2 units per mg
Average of 8 preparations from beef serum 2 2 units per mg

REFERENCE

Loomis E C George C Jr and Ryder A Fibrinolysin nomenclature unit assay preparation and properties Arch Biochem *12* 1 1947

4 Estimation of Antifibrinolysin in Plasma or Serum

E C LOOMIS

Object of the Test To determine the number of units of antifibrinolysin present in plasma or serum

Principle of the Test Serum is added to an excess of fibrinolysin and allowed to react for a standard time The excess fibrinolysin is then determined and the amount of antifibrinolysin is calculated by difference

Reagents and apparatus required (1) Plasma or serum to be assayed (2) Fibrinolysin (Parke Davis and Co) (3) Bovine Fibrinogen, 0 6 per cent clottable in imidazole buffered solution (most Armour and Co lots suitable but preferably prepared by method of Ware et al [1]) (4) Bovine Thrombin (Parke Davis and Co) 50 u /ml in 50 per cent glycerol (5) Open ended capillary tubes (e g melting point tubes) (6) 10 x 75 mm test tubes (7) Pipets (8) Water bath at 45 C (9) Stopwatch

Steps in performance of test (1) If plasma is to be assayed convert it to serum (remove fibrinogen) by recalcification or addition of thrombin Express the serum from the clotted fibrin and assay Any dilution in this step should be corrected for in the calculations below

(2) Into a 10 x 75 mm test tube place at least 30 units of fibrinolysin dissolved in 0 9 ml physiologic saline buffered with imidazole (see page 165) Add 0 1 ml serum to be assayed Allow to stand for 60 minutes at 26–28 C then assay the remaining fibrinolysin

(3) Place 0 3 ml of 50 unit thrombin into a 10 x 75 mm test tube add 0 2 ml of the solution from (2) (or suitable dilution of that solution) Start a stopwatch upon the addition of 0 5 ml of 0 6 per cent bovine fibrinogen As soon as a clot forms place the test tube in a 45 C water bath insert an open end capillary tube to the base of the clot The end point of the determination is reached when the liquid level in the capillary rises to the clot level in the test tube This should be between 90 and 145 seconds or a different dilution should be assayed The fibrinolysin solution used in (2) should also be assayed following the step being described

Calculations

$$A = 10\,(0\,9\,F_O - F_R)$$

$$F = 5\,CF \times D$$

Where A = units of antifibrinolysin per ml of plasma or serum F_O = units of Fibrinolysin per ml of original solution F_R = units of Fibrinolysin per ml after 1 hour reaction C F = correction factor for deviation from

120 second lysis time, D = dilution used for assay, F = units of Fibrinolysin

The correction factor table is printed with the fibrinolysin assay (see page 164)

Normal range of values Normal human plasma or serum contains from 140–165 units of antifibrinolysin per ml

Precautions and sources of error Fibrinogen and thrombin contamination as stated in section dealing with fibrinolysin assay (see page 164)

REFERENCES

[1] Ware A G Guest M M and Seegers W H Fibrinogen with special reference to its preparation and certain properties of the product Arch Biochem *13* 231 1947

[2] Loomis E C George C Jr and Ryder A Fibrinolysin nomenclature unit assay preparation and properties Arch Biochem *12* 1 1947

5 Preparation of Antifibrinolysin

E C LOOMIS

Antifibrinolysin may be prepared from bovine equine and human plasmas by the method to be described Oxalated is preferable to citrated plasmas When serum is used it may be prepared by the method described in the section dealing with the preparation of fibrinolysin (page 165) Temperature control below 26 C is desirable since solubility factors are reduced at lower temperatures Hydrogen ion adjustment on the acidic precipitation is critical and affords only a narrow range between pH 3 5–4 0

Preparation One liter of bovine serum is cooled to +5°C and brought to 0 5 saturation by adding an equal volume of saturated $(NH_4)_2SO_4$ The precipitate is removed by centrifugation in the cold The supernatant treated serum is cooled to +5°C and the degree of saturation increased to 0 70 by the addition of 13 5 g C P $(NH_4)_2SO_4$/100 ml The precipitate is collected by centrifugation in the cold dissolved in 100 ml distilled water and dialyzed 18–20 hr in Visking "No Jax" casing 20/32" against cold running tap or deionized water

The clear dialysis residue is again brought to 0 50 saturation at +5°C by the addition of an equal volume of saturated $(NH_4)_2SO_4$ adjusted to pH 3 75 with N H_2SO_4 the precipitate removed by centrifugation in the cold and the supernatant solution neutralized to pH 7 0 with N NaOH

The neutral solution is again dialyzed as above and dried from the frozen state The antifibrinolysin product obtained is about 85% pure on electrophoretic analysis

Unit One unit of antifibrinolysin has been defined as that quantity which will exactly neutralize one unit of fibrinolysin (see page 166) buffered with imidazole at pH 7 2, in 1 hour at 26°C

Assay An excess of fibrinolysin is dissolved in 0 9 ml buffered physiological saline and 0 1 ml antifibrinolysin solution is added At the end of 1 hour the excess fibrinolysin is determined as previously described and the antifibrinolysin activity determined by difference It has been found that 1 hour is required for the in vitro neutralization of fibrinolysin by antifibrinolysin If the time is extended another 30 minutes less than 10 per cent additional antifibrinolysin is apparent

Reference

Loomis E C Ryder A and George C Jr Fibrinolysin and antifibrinolysin biochemical concentration of antifibrinolysin Arch Biochem *20* 444 1949

CHAPTER XI

TISSUE COAGULANTS*

1 Estimation of Thromboplastin Activity of Tissue Extracts

J F JOHNSON and W H SEEGERS

(a) In Vitro Two-stage Method

Thromboplastin, plus other activators, is essential for the rapid physiologic activation of prothrombin to thrombin in the formation of the blood clot In the body economy it can be supplied by tissue or by a combination of platelets and plasma Thromboplastic agents apparently are widespread in nature many substances supplying a similar activity For analytic work bovine lung thromboplastin has proved a satisfactory and stable source of this material for periods as long as six months for a single preparation Other animal tissues are suitable, such as canine lung, human or rabbit brain each animal and particular tissue varying as to its activity

The preparation of bovine lung thromboplastin has been described elsewhere (page 131) In order to test roughly the activity of the preparation, the material is diluted 5 1 with 0 85 per cent NaCl and tested in a two stage analysis for prothrombin Bovine control plasma is used as the substrate If the usual prothrombin values are obtained the product is acceptable

As a further check the material is made up with 0.85 per cent NaCl at two times this strength and to one-half this strength These additional dilutions are used as was the first and the concentrations of prothrombin obtained from the three analyses are compared If the values are essentially the same, then the original dilution of 5 1 is judged correct and the thromboplastin is used at this strength Should the values vary widely

* The thromboplastins of tissue origin are either in the form of a lipoprotein (e g brain lung saline extracts) or in lipid emulsions (cephalin) Several methods for the preparation of both of these groups of materials may be found in the index under
Preparation of thromboplastin
Preparation of cephalin

another dilution is prepared and tested in a like manner until a satisfactory dilution is attained

If further accuracy of activity measurement is desired then the following test may be done

Reagents The reagents used are the same as those in the usual two-stage Ac globulin analysis (see page 131) The test used is an Ac-G analysis in which all the elements are constant except the thromboplastin The thromboplastin used is diluted to a desired level with 0 85 per cent NaCl and is then incorporated as part of the reaction mixture in the following system

Purified prothrombin solution	3 parts
Thromboplastin suspension	3 parts
Serum Ac globulin or platelet Ac G	2 parts
Calcium chloride (0 70% in saline)	1 part
Acacia (15% in saline)	2 parts
Imidazole buffer (pH 7 2-7 4)	1 part

The concentration of the activators is probably constant and in excess in this mixture The prothrombin is diluted to 6 7 units per ml Equal amounts of this reaction mixture are added to the fibrinogen as in the Ac G analysis and the usual endpoints determined

The analysis is calibrated by means of the standard set of curves for serum Ac globulin as shown on page 139 By comparison of the points plotted from the thromboplastin analysis with this standard family of curves a definite number of units of thromboplastin can be assigned to any suspension tested The arbitrary numbers on each of the curves indicate relative concentrations of the respective activator Thus the curves 2 5 8 16 etc represent 2 5 8 and 16/1000 of a unit of thromboplastin per ml of the reaction mixture when thromboplastin is the variable and an accelerator is present in large quantity

It is possible to use bovine plasma as a source of both Ac globulin and part of the prothrombin for the analysis If this is used the shapes of the curves obtained are comparable to the plasma Ac-G chart as shown in the section on Ac globulin analysis (page 129) If plasma is used it is not necessary to defibrinate but it should be handled in the same way as a plasma is handled in the usual Ac-G analysis

The value of the optimal thromboplastin dilution is that which will give the accepted values of the accelerator in the reaction mixture

Precautions and Sources of Error Thromboplastin dilutions for this analysis should be relatively great due to the display of antithrombin if it is present in the suspension Should this be present the curve for the throm bin values will rise from the base line after attaining an initial low point

This indicates that there is thrombin destruction and the test is not valid To correct this a greater dilution of the thromboplastin is made until the curve remains level after its initial drop

As in the Ac globulin analysis, extreme care must be taken that the prothrombin is free of such activators as Ac G or the natural accelerator that develops in freeze dried prothrombin on long storage Prothrombin *heated to 53°C for one hour in salt 'free' solution, then acetone dried,* is fairly stable and free of Ac globulin

This analysis, like the analysis for Ac globulin, is extremely sensitive to the smallest change in the reagents and because this test is a measure of rate, the slightest variation will be magnified and affect the outcome For this reason the utmost care must be taken in all measurements and pipetting in order to avoid technical error as much as possible

(b) In Vivo Assay (Method of Schneider)

Young white mice are used as the test animals Other experiments with different strains of white mice as well as brown mice and wild mice have shown that they are all suitable as test *subjects* No sex difference has been noted except that during pregnancy the sensitivity increases

Trial doses of the extract to be tested are given intravenously in the *lateral tail veins of weighed mice until the minimum* lethal dose for a 20 gram mouse can be determined or estimated from interpolation curves which have been constructed previously in the testing laboratory by injection of the thromboplastin of different dilutions The toxicity of the material is then expressed as the number of MLD or units per ml Ordinarily about 8 mice will be used for the calibration of an *unknown, after* the preliminary determinations

Light ether anesthesia may be used for convenience during *the* test apparently without disturbing the endpoint The endpoint of the assay is the death of the mouse in a few minutes after the injection usually within two minutes, and not more *than five Some animals may become comatose* and convulse following the test but recover Such a reaction indicates that a near lethal dose has been given Almost always the mice are able to throw off the effects rapidly if the dose is not fatal at once

Injection of the material must be intravenous The lethal dose is increased markedly *if the injection is given slowly or in divided doses* Back flow of blood into the needle or the barrel of the syringe should be avoided

The syringe is filled with the exact dose and after the insertion of the needle the extract is forced into the vein in a single rapid injection

Usually a suitable lethal volume is of the order of 0 10 ml, and in performing the assay the extract is diluted to this activity The dose given to each animal may be increased or decreased by changing the volume of the extract to be injected or by dilution of it or both according to the response of the animal or animals, used in the test Experience is the most valuable guide to the initial dilution and volume A suggested maximum volume of any material is 0 4 ml The assay under proper conditions, is reliable to approximately 10%

A rapid tolerance develops in the animals after the injection of the thromboplastin The animals may be used again for screening assays but they should not be used until after at least three days preferably longer The increased tolerance of the animals to the toxin is at its highest within a few minutes and persists for a few hours at the end of eight hours the animals will have returned to nearly the previous level of sensitivity Similarly complete desensitization can be established by the use of small repeated dosages

The sensitivity of the mouse is not directly proportional to the body weight large or obese mice being more sensitive per gram of body weight Corrections to the lethal dose for mice of 20 grams body weight can be made by comparison with standard curves if desired Unless the variation of weight is extreme the error without correction as compared with mice of 18 to 21 grams is not great

The investigation of the sensitivity of the mouse has also brought out the fact that the animals become more sensitive as they become older and larger The optimum weight is about 18 to 21 grams This represents the body weight of a young healthy laboratory mouse

References

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—— Complications of late pregnancy in rabbits induced by experimental placental trauma Surg Gyn & Obst *90* 613 1950

Thomas L Studies on intravascular thromboplastic effect of tissue suspensions in mice Bull Johns Hopkins Hosp *81* 1 "6 1947

CHAPTER XII

ANTICOAGULANTS IN BLOOD, PLASMA AND SERUM

1 Demonstration of Abnormal Anticoagulant Activity in Blood (Method of Conley, Hartman and Morse)

Adapted by R R HOLBURN*

Object of Method Detection of abnormal anticoagulant activity in the blood by the addition, to normal blood of the test material in the form of native (undecalcified) platelet-poor plasma

Principle Platelet poor plasma from normal individuals invariably clots in glass tubes at 37°C When abnormal anticoagulant activity is present, the addition of this platelet poor plasma in small amounts to normal blood prolongs its clotting time significantly

Apparatus and Reagents Needles, syringes, test tubes and pipets are treated with silicone according to the technique of Jaques (see page 3) A nontraumatic venepuncture is made with an 18 gauge needle After several milliliters of blood to be tested have been withdrawn into a syringe, the needle is left in place the syringe is disconnected, and its contents are discarded This serves to rinse the needle free of any tissue juice A clean silicone coated syringe is then attached and 20 ml of blood are carefully drawn The blood is placed in an icebath in a silicone tube Immediately the tube is centrifuged at about 7000 rpm (6 000 g) at 4°C for 6 minutes to remove the blood cells and most of the platelets The upper portion of the plasma is removed with a silicone coated dropper pipet and recentrifuged at about 12 000 to 14,000 rpm (about 17 500 to 22 000 g) for 10 minutes The upper half of the plasma is removed with a silicone coated pipette and stored in an ice bath until used Normal plasma obtained in this manner may be kept several hours at 4°C without spontaneous coagulation

Steps in Procedure Fifteen scrupulously clean glass tubes (13 x 100 mm) are mounted in a rack and to these are added 0 85 per cent NaCl

From Bulletin Johns Hopkins Hospital 84 255 1949

and platelet-poor plasma as follows (1) 0 1 ml 0 85 per cent NaCl (2) 0 5 ml 0 85 per cent NaCl (3) 0 1 ml plasma (4) 0 2 ml plasma, (5) 0 5 ml plasma (6) 0 5 ml plasma (7) 0 2 ml plasma (8) 0 1 ml plasma (9) 0 5 ml 0 85 per cent NaCl (10) 0 1 ml 0.85 per cent NaCl (11) 0 1 ml 0 85 per cent NaCl (12) 0 5 ml 0 85 per cent NaCl (13) 0 1 ml plasma (14) 0 2 ml plasma, (15) 0 5 ml plasma

With a silicone treated syringe and needle sufficient blood is drawn from a normal individual to add 1 ml portions to each of the fifteen tubes in the order listed A stop watch is started at the time of the venepuncture when the blood first appears in the syringe When the tubes are filled they are shaken uniformly to mix their contents and the rack is placed in a 37 C water bath The clotting time of each tube is recorded the end point is a solid clot

Calculation Significant prolongation of the clotting times in the tubes containing platelet poor plasma is indicative of the presence of abnormal anticoagulant activity Normal platelet poor plasma may occasionally prolong the clotting time of whole blood however this rarely exceeds the clotting time of the 0 85 per cent NaCl controls by more than 7 minutes whereas when abnormal anticoagulant activity is present the clotting time is greatly prolonged When normal serum fresh or stored is used in place of the platelet poor plasma there is no prolongation of the clotting time of whole blood Abnormal anticoagulant activity is usually detectable by the use of platelet poor plasma but in some individuals not necessarily in the serum When abnormal anticoagulant activity is demonstrated its potency may be estimated by determining the smallest amount of platelet poor plasma necessary to prolong the clotting time of normal whole blood

Precautions and Sources of Error A nontraumatic venepuncture and change of syringe to assure the blood is free of tissue juice

Use of silicone surfaces throughout in the preparation of platelet free plasma

2 Estimation of Excessive Anticoagulant Activity

L M TOCANTINS, R R HOLBURN and R T CARROLL

Object of the Method The demonstration of the presence of excessive anticoagulant in unusually stable blood

Principle Plasma containing an excess of anticoagulant prolongs the clotting time of stable normal plasma The least amount of the unknown plasma which causes a significant increase in the clotting time of the normal plasma serves as a measure of the degree of anticoagulant activity in the unknown plasma

Reagents and Apparatus Siliconized 13 mm i d tubes Siliconized pipets graduated at 0 01 ml *Stable plasma* Blood is drawn from a clean venepuncture of a turgid vein using an #18 gauge needle, into a siliconized syringe containing one hundredth volume of 38 per cent sodium citrate Immediately after withdrawal of the blood the needle is removed and the blood and citrate well mixed by inversion By applying gentle pressure to the plunger of the syringe the blood is allowed to run down the wall of a siliconized test tube to avoid bubbling and foaming The citrated blood is centrifuged at 2400 *g* for 50 minutes at 4°C The upper three fourths of the plasma is removed by means of a siliconized dropper pipette, avoiding agitation of the cellular elements Normal and unknown plasmas are prepared in the same way $CaCl_2$ 0 2 M 2 22 grams anhydrous $CaCl_2$, C P dissolved in 100 ml distilled water

Steps in Procedure A series of seven silicone coated test tubes is set up, as shown in table 1 in water bath at 38°C The abnormal plasma is added to the stable normal plasma and the calcium chloride added immediately after The contents are mixed by tilting the tube once and then the tube is placed back into the water bath The tubes remain undisturbed for fifteen minutes Thereafter they are checked for clotting at 100 second intervals When incipient clotting is noted the frequency of removal is increased Too frequent checking of the tubes is undesirable as it tends to shorten clotting times due to the excessive agitation of the contents

Calculation A curve of the data is plotted on semilog paper the clotting times on the logarithmic and the contents of the mixture on arithmetic ruling A significant anticoagulant effect is considered to exist when one part of the unknown plasma is capable of delaying the clotting of no less than three parts of the normal by a value equivalent to one third the clotting time of an equal volume of the whole normal plasma Results are

TABLE I *Testing of Unknown Plasma for Anticoagulant Activity Silicone Coated Tubes 38 C*

Tube	Normal Plasma (ml)	Unknown Plasma (ml)	0 2 M CaCl (ml)	Clotting Time (secs)	Normal Plasma %	Unknown Plasma %
1	0 5	0 0	0 05	1 850	100	0
2	0 45	0 05	0 05	2 850	90	10
3	0 4	0 1	0 05	3 250	80	20
4	0 3	0 2	0 05	4 400	60	40
5	0 2	0 3	0 05	6 500	40	60
6	0 1	0 4	0 05	41 000	20	80
7	0 0	0 5	0 05	No Clot at 24 hrs	0	100

expressed in terms of the proportion of unknown to normal plasma which can still delay the coagulation of the latter significantly For example in table 1 one part of the unknown can delay significantly the rate of clotting of nine parts of normal plasma This proportion has been observed in certain instances to be as high as 1 in 250

Precautions and Sources of Error (1) The test must be carried out in silicone tubes since plasma that has a clear clot-delaying activity when tested in silicone coated tubes may fail to do so in uncoated glass If there is a great excess of anticoagulant present the clot-delaying effect may be observed even in glass tubes while in silicone even a low content of anticoagulant will exert a clot decelerating effect (2) The normal plasma used as a substrate must be stable when tested in a mixture such as indicated in Tube No 1 in table 1 it should have a clotting time ranging from 1200 to 2400 seconds A normal plasma that is prepared according to the directions given usually proves satisfactory (3) The plasma mixtures must have a high final plasma concentration even after $CaCl_2$ has been added in the mixture named in table 1 the final plasma concentration in each tube is about 87 per cent

REFERENCES

Tocantins L M Holburn R R and Carroll R T The nature of the defect in coagulation of hemophilic blood Trans of the Fourth Conf on Blood Clotting and Allied Problems of the Josiah Macy Jr Foundation 1951 p 82

Tocantins L M Carroll R T and Holburn R R Antithromboplastin activity of the plasma of animals exposed to ionizing radiation Trans of the Fifth Conf on Blood Clotting and Allied Problems of the Josiah Macy Jr Foundation 1952 p 247

3 Estimation of Plasma Antithromboplastin Activity (One-stage Method)

L M TOCANTINS and R R HOLBURN

Definitions *Antithromboplastin* is a term to designate an activity, existing probably in the form of a lipoprotein in the tissues blood plasma and serum, directed against the formation and action of thromboplastin *Lipid antithromboplastin* is the heat stable lipid extracted from the tissues blood plasma or serum, which together with a plasma cofactor is capable of reducing the clot accelerating action of thromboplastin (for preparation and assay see page 221)

Object of the Method To estimate rapidly the degree to which a given plasma can reduce the clot accelerating activity of a cephalin suspension

Principle Citrated plasma collected with especial precautions is incubated with a cephalin suspension for a fixed period of time The delay in the rate of clotting caused by the incubation is compared with that caused by dilutions of the cephalin suspension tested on pooled plasma The loss in cephalin activity is transposed into units of antithromboplastin activity

Apparatus and Reagents Unless otherwise stated silicone surfaces are used throughout in the collection, separation measurement storage and testing of plasma Plasma samples are tested on the same day of collection, frozen specimens are not used since, after thawing they become hypercoagulable Storage of plasma in glass tubes is likewise avoided, since glass like clay asbestos and similar surfaces reduces, and eventually eliminates depending on the extent and duration of contact most of the activity

1 Cephalin Suspensions of cephalin are used as sources of thromboplastin instead of saline tissue extracts because (a) Antithromboplastin activity seems to be directed against the cephalin moiety of the thromboplastin lipoprotein (anticephalin activity) (b) The great lability of saline tissue extracts as concerns thromboplastin potency even after addition of antioxidants and other protective agents makes them unreliable for these assays The spontaneous decrease in activity on simple standing is sometimes considerable and variable among different preparations (c) The stability of cephalin preparations which makes it possible to standardize their activity with assurance of only minor changes in potency over several days (d) As slow activators of prothrombin conversion cephalin suspensions are more vulnerable to the action of inhibitors even when these are in low concentration in the clotting mixture (e) Bacterial contamination of its suspensions is uncommon while in saline tissue extracts bacteria grow rapidly

Preparation of cephalin suspension To 3 0 grams of acetone dried brain powder are added 75 ml of peroxide-free ether The mixture is allowed to extract 24 hours at 5 C The supernatant liquid is decanted through a double thickness of filter paper and concentrated to a volume of 6 to 8 ml in a vacuum desiccator and transferred to a 50 ml tube Cold absolute ethanol is added slowly to the tube until the resulting precipitate appears less concentrated at the top This is the point of maximum precipitation Further addition of ethanol will cause the cephalin to assume a colloidal state and the fine particles will not settle The precipitate is allowed to settle at 5 C until the supernatant is clear (about 30 minutes) The suspension is centrifuged 2 minutes at 1000 rpm and the supernatant discarded The precipitate is redissolved in 4 to 5 ml of ether and the alcohol precipitation repeated A preparation precipitated six to eight times is almost free of inhibitors and is suitable for use

After the final supernatant ethanol is discarded, the precipitate is washed into a small weighed beaker by as small a volume of acetone (10 ml maximum) as possible The acetone is decanted and discarded when the precipitate has settled The beaker is rotated in the hand until the odor of acetone is no longer evident The powder should be white not yellow and yield about 3 per cent by weight of the starting material The cephalin is immediately weighed and then suspended in a 0 85 per cent NaCl solution while scraping the sides and bottom of the beaker with a glass rod The final concentration of the suspension should be 3 per cent A hand homogenizer is effective in making a finer dispersion of the cephalin particles The suspension should then be heated at 65 C for ten minutes in case the preparation should be hyperactive because of thromboplastic lipoprotein contaminants The saline suspension will keep at least a month at 5 C and the cephalin powder may be stored indefinitely under ethanol without loss of potency

The cephalin preparation is tested for the presence of inhibitors by noting the clot accelerating action of the suspension as it is progressively diluted With crude preparations the initial dilutions accelerate coagulation more than the undiluted suspension with purified preparations any dilution even though small results at once in a decrease in activity

In order to test the suspension for freedom from inhibitors the following clotting mixture is made 0 1 ml cephalin (3 per cent) 0 1 ml citrated plasma 0 1 ml 0 02 M $CaCl_2$ added in the order named since if the $CaCl_2$ is added after the cephalin suspension it will flocculate it and vitiate the results A typical example of the testing follows

Tube No	3% Cephalin Suspension (ml)	0 85% NaCl (ml.)	Plasma (ml)	0 02 M CaCl (ml)	Clotting time (sec)	
					2 X ppted Cephalin	8 X ppted Cephalin
1	0 1	—	0 1	0 1	180	135
2	0 05	0 05	0 1	0 1	150	150
3	0 03	0 0"	0 1	0 1	140	166
4	0 015	0 085	0 1	0 1	135	198
5	0 01	0 09	0 1	0 1	140	220

The 2x precipitated cephalin preparation is not suitable for assay of antithromboplastin

2 Stable plasma Blood from a neat, fast venepuncture from which the first few milliliters have been discarded is drawn into a silicone coated syringe containing an anticoagulant (0 1 ml 38 per cent sodium citrate per 10 ml blood) The blood and anticoagulant are well mixed in the syringe and then run down the side of the silicone tube with gentle pressure on the plunger of the syringe After centrifuging at 5°C for 60 minutes at 2000 g, the upper four fifths of the plasma is removed with a silicone coated dropper pipet and stored at 5 C for testing the same day Blood from a normal control is collected by the same technique at the same time

3 $CaCl_2$ 2 22 Gm anhydrous $CaCl_2$ is dissolved in 100 ml distilled water (0 2 M)

Steps in the Procedure Place 0 1 ml of a 1 per cent purified cephalin suspension in each of two 13 mm wide silicone coated tubes To each add 0 5 ml of the citrated plasma To the first tube add at once 0 05 ml 0 2 M $CaCl_2$ shake stopper the tube and measure the clotting time in a water bath at 38 C Shake and stopper the second tube and incubate at 38°C for 30 minutes then add 0 05 ml of 0 2 M $CaCl_2$ and measure the clotting time

All glass surfaces (tubes pipets) coming in contact with plasma are siliconized

Calculation In order to convert the clotting times into units of antithromboplastin (anticephalin) activity the response of a pooled stable normal plasma (no less than 5 in the pool) to a standard suspension of cephalin must be worked out This is done by adding to this plasma gradually decreasing amounts of a cephalin suspension, the potency of which in units has been determined (See two-stage method of measuring antithromboplastin activity page 185) Let us say that the 1 per cent suspension of cephalin is known to have 90 units of cephalin activity per mgm Dilutions of this suspension are tested on the pooled plasma for their clot accelerating activity until they equal the clotting time of the control (0 85

per cent NaCl instead of cephalin) as follows

Tube No	1% Cephalin Suspens	0 85% NaCl (ml)	Plasma (ml)	0 2 M CaCl (ml)	Units of Cephalin activity in mixture	Clotting Time (sec)
1	0 1	0	0 5	0 05	180	315
2	0 075	0 025	0 5	0 05	135	370
3	0 05	0 05	0 5	0 05	90	460
4	0 025	0 075	0 5	0 05	45	660
5	0 016	0 084	0 5	0 05	30	805
6	0 008	0 092	0 5	0 05	15	1175
7	0	0 1	0 5	0 05	0	1180

In the table above the cephalin activity is expressed as units added per *1 ml* plasma this facilitates in converting the antithromboplastin activities from *0 5 ml* (amount used in the clotting mixture) to 1 ml (the amount used in reporting the results) The results clotting times as ordinates and units of cephalin activity as abscissa plotted in log/log paper yield a straight line as shown in figure 1 The clotting times of the unincubated and the incubated plasmas are then located on the line and the correspond

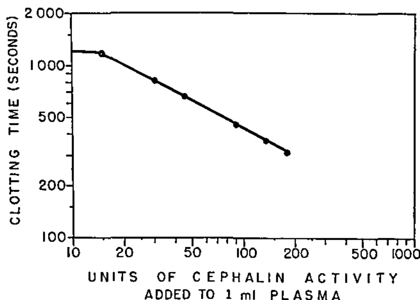


FIG 1 —Standard curve for one stage estimation of cephalin activity—pooled normal plasma

ing units of cephalin activity are read directly below in the abscissa The units of cephalin activity lost during incubation are obtained by subtracting the units corresponding to the clotting time of the incubated plasma from those of the unincubated One unit of antithromboplastin (anti cephalin) activity is by definition, that activity of the plasma required to destroy one unit of cephalin activity in silicone coated tubes after 30 minutes of incubation at 38 C of the plasma and the cephalin suspension The units of cephalin activity lost are therefore directly converted to units of antithromboplastin activity

Example Three plasmas, one normal, one hypercoagulable and one hemophilic were tested as described The clotting times and the calculated results follow

Type of Plasma	Clotting Time (secs)		Equivalent Cephalin Units		Antithromboplastin Activity Units
	Unincubated	Incubated	Unincub	Incub	
Normal	420	460	107	90	17
Hypercoagulable	315	325	180	175	5
Hemophilic	635	1000	68	20	48

Range of Values Mean of 33 determinations performed on 33 normal men and women 14 8 antithromboplastin units per ml plasma ± 4 2 (stand dev) The mean value for 20 determinations on 13 Hemophilia A patients was 47 2 units per ml plasma ± 13 8 (stand dev)

Precautions and Sources of Error (1) Defectively collected plasmas are unsuitable for testing because the evolution of accelerators during partial clotting offsets the action of inhibitors (2) The plasma must not be frozen or allowed to stand longer than 3 hours at 5°C before being tested (3) The platelet content of the plasma must be less than 4 000 per cm (4) The ratio of 5 parts of plasma to 1 of cephalin must be maintained in the incubation mixture since dilution of the plasma will impair its inhibitor potency Most tests for antithromboplastin described in the literature overlook this precaution (5) For best results the cephalin suspension should be prepared from brain tissue of the same species as that of the plasma being tested (6) Adherence to the order of addition of the reagents is essential (7) If the content of the Ac G or prothrombin in the plasma is low (below 25 per cent of normal) the results are unreliable For example if the plasma of a hemophilic A patient contains only 20 per cent normal prothrombin, the incubated mixture may not clot at all, making it impossible to read the values on the cephalin standard curve chart

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4 Demonstration of Thromboplastin-Inhibiting Activity in Serum and Plasma (Method of Lanchantin and Ware)

Adapted by R R HOLBURN*

Principle When barium sulfate adsorbed serum or plasma is incubated with thromboplastin and calcium chloride a marked inhibition of thromboplastin activity results

Apparatus and Reagents *Saline imidazole buffer* Dissolve 3 Gm Imidazole C P in 975 ml of 0 7 per cent NaCl solution and 25 ml of 0 5 N HCl pH of this solution is 7 4

Thromboplastin Acetone dried human brain powder is prepared by standard techniques Five gms brain powder are extracted with 100 ml of 0 9 per cent NaCl containing 0 002 M potassium oxalate The extraction is carried out at 45 to 50 C for 30 with occasional stirring Light centrifugation removes the gross particles The crude product is centrifuged for two hours at 28 000 g The pellet is resuspended in oxalated saline to its original volume This is repeated twice more After the last resuspension the sediment is taken up in saline imidazole buffer to 10 times its original volume

Prothrombin free beef plasma Freshly collected beef blood is added to 0 1 M potassium oxalate (ratio 9 to 1) After centrifugation the plasma is mixed with freshly precipitated barium sulfate Equimolar solutions of barium chloride and sodium sulfate are mixed and the precipitate is washed twice with distilled water Two grams of the centrifuge packed barium sulfate (dry weight) are added to each 100 ml of plasma dispersed and

From Journal of Clinical Investigation 32 381 1953

allowed to stand at room temperature for 30' The barium sulfate is removed by centrifugation and each 100 ml of plasma run through a Seitz filter of 40–50 per cent asbestos content The plasma is dialyzed against 0.85 per cent NaCl until free of oxalate and then is diluted with an equal volume of 0 9 per cent NaCl containing 0 6 per cent imidazole The pH is adjusted to 7 4 with 0 5 N HCl and the material is frozen

Water bath 37°C All reagents are preheated to 37°C

Steps in Procedure To 75 x 10 mm test tubes are added 0 1 ml prothrombin free beef plasma 0 1 ml reconstituted dried human plasma (1 to 10), 0 1 ml buffer, 0 1 ml thromboplastin 0 1 ml 0 015 M $CaCl_2$ The clotting time is recorded

Unknown samples are substituted for buffer In incubated studies the thromboplastin, calcium and serum are incubated together before the addition of the other two components

Calculation In the absence of thromboplastin the system clots in about 500 seconds When thromboplastin is added the system clots in about 30 seconds Dilution of thromboplastin results in progressively longer times The logarithm of the thromboplastin concentration in this system is a straight line function of the negative logarithm of the clotting time

Incubation of thromboplastin, calcium and (a) normal human serum results in an acceleration phase in the first few minutes which is then followed by inhibition (b) barium sulfate adsorbed human serum results in inhibition immediately, and has the capacity of inactivating 95 per cent of the thromboplastin (c) barium sulfate adsorbed plasma reacts similarly to serum except that accelerators mask the effects somewhat

Precautions and Sources of Error (1) Accelerator factors are more efficiently removed with barium sulfate when oxalate is present Therefore oxalate is added to a strength of 0 02 M and freshly precipitated barium sulfate is added to a concentration of 2 Gm per 100 ml of serum (2) When calcium is omitted from the incubation phase the inhibition of thromboplastin is much less (3) Better success has been achieved in demonstrating inhibition when oxalate free systems are used

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5 Estimation of Antithromboplastin (Anticephalin) Activity in Plasma (Two-stage Method)

R R HOLBURN and L M TOCANTINS

Object To assay the antithromboplastin activity of normal and ad normal plasmas using cephalin as a thromboplastin agent and the two-stage method of estimating thrombin output as a measure of thromboplastin activity

Principle Properly collected plasma has the property of reducing the clot accelerating activity of cephalin after the latter is incubated with the plasma The change in the rate of thrombin output before and after the incubation period will reflect the loss of cephalin activity Using a standard curve of cephalin activity as reference clotting times are converted into units of antithromboplastin

Apparatus and Reagents (Siliconized glassware used throughout except in step 3 of the procedure when fibrinogen is added and uncoated glassware may be used) (a) *Stock thrombin solution for defibrinating* Dissolve the contents of one ampoule (1000 units) of thrombin (Upjohn Co) in 2 ml of distilled water Add an equal volume of glycerol C P The final concentration of this solution should be 250 units per ml (b) *Acacia Solution* Dissolve 15 grams of powdered U S P acacia in 100 ml of 0 85 per cent NaCl solution Centrifuge lightly to remove a small amount of the insoluble material In general commercial acacia contains about 0 6 per cent calcium by weight (0 023M) It is diluted when added to the incubation substrate to bring its calcium concentration into optimum range (0025M to 01M) (c) *Imida ole buffer* (pH 7 2–7 4) Dissolve 1 72 grams imidazole (Eastman Kodak) in 90 ml of 0 1N HCl and dilute to 100 ml with water (d) *Fibrinogen* 50 mg of fibrinogen (prepared as described on page 157) is dissolved in 5 ml of buffered saline solution (0 85 per cent NaCl containing 5 per cent imidazole by volume) Centrifuge lightly after thawing to remove any insoluble material (e) *Activation substrates* 2 parts of 15 per cent acacia in 0 85 per cent NaCl ½ part imidazole buffer 3 ½ parts 0 85 per cent NaCl (f) *Cephalin* The purified preparation from human brain powder as for the one stage antithromboplastin test (see page 178) and used in 1 per cent concentration (g) *Stable Plasma* Collected with especial precautions as described on page 176 Siliconized syringes and tubes (h) *Standard Control Plasma* Lots of 50 or more small quantities of pooled normal plasma are frozen and used as a standard

control for reagent reactivity After thawing at 37°C this plasma is treated subsequently in the same manner as the freshly collected plasma

Steps in Procedure (1) To 0 1 ml 1 per cent cephalin add 0 9 ml defibrinated plasma in a siliconized tube (2) Mix and remove 0 1 ml of the mixture and add to a siliconized tube containing 1 5 ml buffered 0 85 per cent NaCl and 3 0 ml Incubation Substrate Start stop watch Stopper remainder of the plasma cephalin mixture and allow to incubate 30 minutes at 38°C (3) At three to four minute intervals remove 0 3 ml of the activated mixture and add to 0 075 ml 1 per cent purified fibrinogen solution Record clotting time The *endpoint* is the appearance of heavy flocculation in the system Aliquots are removed and added to fibrinogen until the clotting time of three successive determinations remains the same At the end of the thirty minute incubation period, remove 0 1 ml of the plasma cephalin mixture and proceed as in steps two and three It is not necessary to incubate the standard frozen control plasma with cephalin since inclusion of this plasma in the testing is merely a control to reagent reactivity

If the test plasma should have a prothrombin content of less than 50 per cent, the clotting times of the activated mixture may become inconveniently prolonged It is desirable then to diminish the dilution of the plasma by the activation substrate buffered 0.85 per cent NaCl mixture from 1 to 46 to 1 to 16 This is accomplished by adding 0 3 ml of plasma to 1 5 ml saline and 3 0 ml incubation substrate this will reduce the total dilution from 70 1 to 23 4 and the clotting time from 39 seconds to 19 seconds, for a plasma of 30 per cent prothrombin concentration Changing the dilution factor does not alter the plasma (antithromboplastin) activity determination If the Ac globulin content of the plasma should be reduced below 20%, there will be some reduction in the thrombin yield, however a minor change in the dilution factor will result in a satisfactory decrease in the clotting time, without affecting the rate of thrombin output, since the comparison of the decrease in rate of thrombin formation of the nonincubated and incubated specimens is the basis of measurement of antithromboplastin activity

Calculation The curves expressing the rate of conversion of prothrombin to thrombin of the various samples are plotted on arithmetic ruled paper The times at which the aliquots were removed from the reacting mixture are plotted along the abscissa the amount of thrombin formed at each sampling along the ordinate axis

The amount of thrombin formed is calculated on the basis that 1 unit of thrombin is that amount which clots the fibrinogen solution in 15 seconds under the conditions of the test (table 1) The factor thus obtained is multiplied by the total dilution to give the total thrombin units per ml of

TABLE 1 *Conversion Factors for Calculation of Thrombin Unitages*

Seco ds	F tor	Seconds	F cto	Seconds	Facto	Seconds	Facto
11 0	1 50	15 0	1 00	19 0	75	23 0	65
2	1 47	15 2	97	19 2	74	23 2	64
4	1 44	15 4	96	19 4	73	23 4	64
6	1 41	15 6	95	19 6	73	23 6	64
8	1 38	15 8	94	19 8	72	23 8	64
12 0	1 34	16 0	92	20 0	72	24 0	64
2	1 31	16 2	91	20 2	72	24 2	63
4	1 28	16 4	89	20 4	71	24 4	63
6	1 25	16 6	88	20 6	71	24 6	63
8	1 23	16 8	86	20 8	70	24 8	61
13 0	1 20	17 0	85	21 0	70	25 0	60
2	1 17	17 2	84	21 2	69	26 0	49
4	1 16	17 4	83	21 4	68	27 0	47
6	1 13	17 6	82	21 6	68	28 0	44
8	1 12	17 8	81	21 8	68	29 0	43
14 0	1 10	18 0	80	22 0	67	30 0	40
2	1 07	18 2	79	22 2	66	31 0	38
4	1 05	18 4	77	22 4	66	33 0	34
6	1 03	18 6	76	22 6	65	35 0	31
8	1 02	18 8	76	22 8	65	37 0	28

plasma The dilution factor is obtained as follows $\frac{59}{60}$ (anticoagulant based on a hematocrit of 40) $\frac{100}{108}$ (defibrination) $\frac{9}{10}$ (cephalin), $\frac{1}{16}$ (saline) $\frac{16}{46}$ (activation substrate) $\frac{300}{375}$ (fibrinogen)

$$\frac{59}{60} \times \frac{100}{108} \times \frac{9}{10} \times \frac{1}{16} \times \frac{16}{46} \times \frac{300}{375} = \frac{1}{70\ 17}$$

The estimation of the thrombin yield per minute is made from the curves for prothrombin conversion (fig 1) The data presented in table 2 is shown in the curves plotted in figure 1 The rate of thrombin output per minute is obtained by dividing the maximum thrombin yield for each plasma by the time in minutes required to reach this value

$$\frac{\text{Total thrombin formed (units/ml plasma)}}{\text{Minutes required for complete conversion}}$$

$$= \text{Thrombin units/ml plasma/minute}$$

From the curves on figure 1 the thrombin units/ml plasma/minute

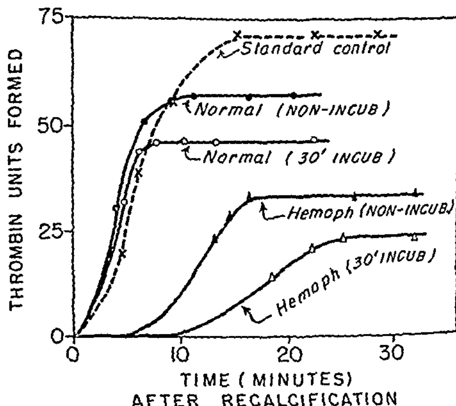


Fig 1—Anticephalin activity of normal and hemophilic plasmas The rate of prothrombin conversion in (a) standard control normal plasma (b) normal plasma non incubated and (c) incubated with a cephalin suspension before recalcification (d) Hemophilia A plasma non incubated and (e) incubated with a cephalin suspension before recalcification

From the above curves the rate of thrombin output per minute is calculated for each plasma The incubated plasmas form less thrombin and more slowly than the nonincubated ones The chief object of the standard plasma is to provide a reference control for the reactivity of reagents from day to day

are as follows

$$\text{Control Plasma } \frac{71\ 5}{14} = 5\ 1$$

This result must be corrected by a factor for reagent reactivity since by definition the control plasma should produce 5 6 thrombin units/ml plasma/minute

Correction factor

$$\frac{\text{Thrombin units/ml standard frozen plasma/min by definition}}{\text{Thrombin units/ml standard plasma/min. in the current test}} = \frac{5\ 6}{5\ 1} = 1\ 1$$

TABLE 2 *Time of Sampling and Equivalent Clotting Times and Thrombin Units Developed by the 5 Plasmas*

Type of Plasma	Time of Sampling (Minutes)	Clotting Time (Seconds)	Conversion Factor	Dilution	Thrombin Yield (Units)
Frozen Control	4	37.0	28	× 70 1	20
	6	25.8	50	× 70 1	35
	8	17.6	82	× 70 1	57
	14	14.8	1.02	× 70 1	71.5
	20	14.8	1.02	× 70 1	71.5
	28	14.8	1.02	× 70 1	71.5
Normal nonincubated	4	29.0	43	× 70 1	30
	7	19.2	74	× 70 1	52
	10	17.8	81	× 70 1	57
	16	16.8	86	× 70 1	60.3
	20	16.8	86	× 70 1	60.3
Normal incubated 30	5	27.8	45	× 70 1	31.5
	7	25.8	52	× 70 1	36.5
	8	25.4	56	× 70 1	39.3
	10	25.2	59	× 70 1	41.3
	14	25.2	59	× 70 1	41.3
	22	25.2	59	× 70 1	41.3
Hemophilic nonincubated	10	60	—	—	—
	12	34.0	33	× 70 1	23.2
	14	30.5	39	× 70 1	27.5
	16	25.7	51	× 70 1	35.8
	26	25.7	51	× 70 1	35.8
	32	25.8	51	× 70 1	35.8
Hemophilic incubated 30	10	60	—	—	—
	18	42.0	19	× 70 1	13.4
	22	36.0	29	× 70 1	20.5
	25	33.0	34	× 70 1	24.0
	32	33.2	33	× 70 1	23.1

This factor is used to correct the results obtained in the testing of the fresh plasmas as follows

Normal plasma (nonincubated) $\frac{60.3 \times 1.1}{11} = 6.0$

Normal plasma (incubated) $\frac{41.3 \times 1.1}{9} = 5.0$

Hemophilic plasma (nonincubated) $\frac{35.8 \times 1.1}{12} = 3.3$

Hemophilic plasma (incubated) $\frac{23.1 \times 1.1}{25} = 1.0$

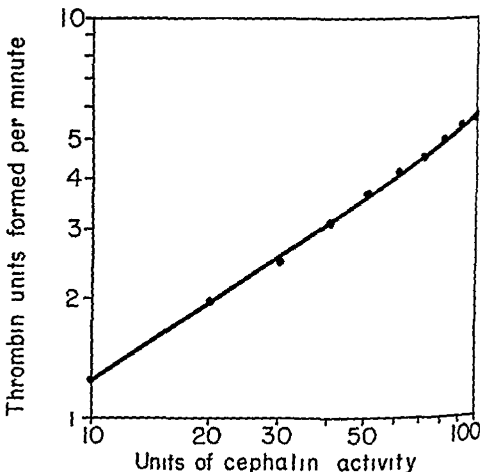


FIG 2—Standard curve for two stage estimation of cephalin activity

The next step is to convert the thrombin units per ml plasma per minute into units of cephalin activity This is done by referring to the standard chart (fig 2) which expresses this activity in terms of thrombin units per ml plasma per minute

Derivation of the Standard Curve of Cephalin Activity A 1 per cent purified human cephalin suspension is diluted with 0 85 per cent NaCl solution from 1 0 to 0 1 per cent concentration A 0 1 ml aliquot of each dilution is added to 0 9 ml of the defibrinated standard control plasma (kept frozen) and the two stage test procedure followed The values for the rate of thrombin formation (thrombin units/ml plasma/min) are plotted on log log paper on the ordinate and on the abscissa the mg of cephalin added per ml plasma along with the coincident cephalin activity as defined Cephalin activity is expressed in units, one unit being that

amount of cephalin which under the conditions of the test will activate standard frozen normal plasma to produce a maximum yield of 0 062 T U /ml plasma/min The line representing the relation between thrombin output and cephalin activity is shown in figure 2 and is the result of testing of four separately prepared cephalin suspensions Since the values for each of the four lines were superimposed on one another the line on figure 2 representing the mean values for the four, was adopted as the standard of reference to express the activity of the cephalin suspensions

Employing the chart in figure 2 as a reference standard the cephalin activities of the plasmas the curves of which are shown on figure 1, are

Normal plasma (unincubated) *95* units
Normal plasma (incubated) *77* units
Hemophilic plasma (unincubated) *50* units
Hemophilic plasma (incubated) *6* units

The units of antithromboplastin (anticephalin) activity are the difference between the units of cephalin activity of unincubated and incubated plasmas

Normal (0 incub) − Normal (30 incub) = 95 − 77 = 18 units

Hemophilic (0 incub) − Hemophilic (30 incub) = 50 − 6 = 44 units

The frozen control plasma functions as an index to reagent reactivity in addition to its primary purpose of having supplied the substrate for the standardization of the cephalin suspensions When this plasma is about exhausted a new one is collected divided into small lots (2 ml) and stored at −10 C The response of the new plasma standard to a cephalin suspension is compared and adjusted to that of the old plasma

Cephalin suspensions prepared as described have been uniform in activity when kept at 5 C for over three months The average rate of thrombin production per ml of plasma (stored frozen) per minute activated by 1 1 mgm of cephalin is 6 2 thrombin units this has been regarded to represent 100 units of cephalin activity

One unit of cephalin activity is therefore that amount which will activate a standard plasma to produce 062 thrombin units per ml plasma per minute One unit of antithromboplastin (anticephalin) activity is that which after incubation of the plasma at 38°C for 30 minutes as described will reduce by 062 units the capacity of the cephalin suspension to activate the conversion of prothrombin in a plasma Or in other terms one unit of antithromboplastin is that amount which will inactivate one unit of cephalin activity

The fundamental criterion in the evaluation of inhibitory activity of plasma is the stability of reagents Cephalin if prepared as described in this technique has the property of maintaining its clot accelerating activity

when incubated alone for 30 minutes at 37°C while some thromboplastin preparations do not Cephalin has additional advantages in that it can be purified, weighed to insure exact concentration and stored at 5°C without loss of activity for several months

A properly collected plasma kept away from glass surfaces is essential for reliable results, since unstable plasma will lead to rapid conversion of prothrombin to thrombin

It is necessary to keep the ratio of cephalin to plasma 1 to 9 in order to have high inhibitor concentration in the plasma cephalin mixture during incubation

Values Obtained In 39 determinations on 39 normal adults the mean antithromboplastin activity by this two stage method was 12.8 ± 4.3 (stand dev) units In 24 tests on 13 hemophilic patients the mean value was *51 0 ± 16 4 (s d) units*

Precautions and Sources of Error (1) Contact of plasma with glass or adsorbants i e asbestos clay results in a hypercoagulable type of plasma, in which after sufficiently long period of contact, it may not be possible to distinguish between the antithromboplastin activities of hypercoagulable normal and hemophilic plasmas This is true also of plasmas resulting from poor venepunctures (2) Prothrombin and ac globulin content should be kept within a practical range by dilution during the measurement of thrombin activity in order to avoid unduly prolonged clotting time

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6 Preparation and Assay of Blood Antithromboplastin

R T CARROLL and L M TOCANTINS

Reagents and Apparatus—These are the same as described for the extraction and assay of tissue antithromboplastin (page 223)

Extraction The blood is withdrawn swiftly (rate of withdrawal no less than 0 5 ml blood per second) from a well distended vein through a #18 gauge needle into a siliconized syringe When the correct volume has been removed the syringe is detached from the needle and the blood added directly to a large uncoated Erlenmeyer flask containing absolute methanol the amount having been previously adjusted to correspond to forty times the volume of the blood sample removed A quick accurate venepuncture is essential thereby eliminating as much as possible the possibility of contamination of the blood with tissue juices The blood should be squirted directly into the methanol and not allowed to touch the glass walls of the vessel before mixing with the reagent

As the blood is added the flask is continuously swirled gently, to avoid ' caking of the blood in the alcohol and the resulting decrease in surface area exposed for the extraction The final mixture should contain fine granular small reddish particles which disperse evenly throughout the solution on gentle swirling The mixture is now allowed to remain at 5 C for a period of 5–7 days Once daily during this period the flask is removed from the refrigerator and its contents mixed by swirling in a rapid rotating motion for a period of approximately two minutes At the end of the extraction period the mixtures are filtered through two thicknesses of Whatman #2 filter paper The residue is rinsed with two 125 ml portions of absolute methanol The filtrate is then placed in a distilling flask immersed in a water bath maintained at 45–48 C The residue remaining in the distilling flask upon removal of all the methanol consists of an aqueous and a solid pha e To the distilling flask is added absolute ethyl ether in 20–25 ml volumes the flask is shaken for three minutes and the mixture placed in a separatory funnel Addition of ether to the flask is repeated until no residue is left in the distilling flask This usually requires between 80 and 100 ml of absolute ether The ether residue mixture is allowed to remain at room temperature for twenty minutes This permits the separation of two phases a clear yellow green upper layer and a dark green almost black lower layer The lower layer is removed with approximately the lower tenth of the upper layer This is then shaken in a separatory funnel with 30 ml absolute ether Again after a 20-minute wait the

lower layer is removed in a similar fashion and the upper layer is combined with the first upper layer sample This process is repeated once more and the combined upper layers placed in a weighed beaker and the ether solu tion removed in vacuo Allowing the ethyl ether methanol residue mix tures to stand in the separatory funnel at 5°C for longer periods results in no appreciable difference in the quantity or quality of the lipid inhibitor This is in contrast to the brain methanol extracts which contain material somewhat soluble in ether at 15 20° but not soluble at 5°C, which exert a coagulant action when tested on plasma

Testing of Extract If it is desired to test the material directly, the beaker is first weighed accurately and the weight of the residue obtained by subtracting the initial weight of the beaker To the product is added sufficient 0 85% NaCl to prepare a 1 or 2 per cent solution In these con centrations, the unknown blood residues are conveniently prepared and homogenize well Usually the gravimetric yields are so small (unless large volumes of blood are initially employed) that the final residue obtained, when mixed with the proper amount of 0 85 per cent NaCl, does not lend itself to homogenization with the supersonic vibrator which requires a minimum volume of 5 ml The residue is mixed well with the saline solu tion using a rod The blood residues are much more easily dispersed in 0 85 per cent NaCl than the tissue residues The pH of the lipid suspen sions is then adjusted and they are passed through the hand operated homogenizer 5 times and exposed to the supersonic vibrator for a period of 20 minutes The blood extracts, when suspended in saline solution and homogenized, retain their state of fine dispersion for a longer period than does the tissue inhibitor, possibly indicating the presence of a suspension stabilizing substance in the blood extracts not usually found in the tissues If the unknown residue is required to stand over a period of 6–8 hours before being tested it is rehomogenized by exposure to the supersonic vibrator for a period of 20 minutes

The testing mixture is essentially the same as that described in testing the tissue inhibitor i e, 0 1 ml unknown fraction 0 1 ml thromboplastin, 0 1 ml normal citrated plasma 0 1 ml 0 02 M $CaCl_2$ added in the order named

The thromboplastin solution used is the same as that described in the assay of the tissue inhibitor (page 223) taking care to use a thromboplastin from the same species as that of the inhibitor and the plasma used as a substrate Since the antithromboplastin obtained from blood is less potent than that obtained from tissues a better assay of the inhibitor results if a thromboplastin less potent than that used for assay of the tissue inhibitor is used Dilution of the brain thromboplastin 1 to 20 and possibly 1 to 30

with 0 85 per cent NaCl makes it possible to obtain a better quantitative differentiation of the various fractions tested

The plasma used in the testing mixture is obtained in the manner described for that used in testing the tissue inhibitor, care being taken not to use a plasma sample that is older than 12 hours Close attention must also be paid in this assay to the amount of $CaCl_2$ added to the citrated plasma to reach the optimal clotting rate It is important that this is found for each lot of plasma being used as a substrate, since both over or under recalcification may vitiate the results If native blood or plasma is being extracted there will of course not be any citrate in the extracts If citrated or oxalated blood is the source provision must be made in the testing to offset the effect of any citrate or oxalate carried through the extraction The technic of determining the calcium concentration required for optimal clotting is as follows Using as reagents those already described a mixture consisting of

0 1 ml suspension of extract
0 1 ml thromboplastin
0 1 ml normal citrated plasma
0 1 ml $CaCl_2$ (of varying molarity)

is prepared The molar concentrations of $CaCl_2$ employed are usually 0 01, 0 0125 0 015 0 0175 0 02 0 0225 0 025 0 0275 0 03 0 0325 0 035 The concentration which yields the shortest clotting time is considered as optimum for that clotting mixture

Assay of the blood extract is carried out exactly as described for the tissue lipid antithromboplastin (page 225) By comparing the clotting time of the unknown with that of the standard the number of units of antithromboplastin activity per mgm of the extract may be found To find the total unitage the units per mgm are multiplied by the yield in mgms The total units divided by the number of ml of blood used will give the units per ml of blood

Example 20 ml of blood were extracted with 800 ml of absolute methanol The final residue weighed 37 mg A 1 per cent suspension was prepared and gave a clotting time in the activated clotting mixture of 66 seconds One mg of the extract being assayed contained therefore 1 96 units in terms of the activity of the reference standard The total activity of the extract was 1 96 $\times$ 37 = 72 5 units

$\frac{72\ 5}{20} = 3\ 62$ units of antithromboplastin activity per ml of blood

With every extraction of an unknown blood sample an extraction of normal blood is performed Every step including the testing is carried out

simultaneously on both samples The activity of the unknown may then be expressed in terms of per cent of normal *Example*

Units of antithromboplastin activity per ml normal blood 1 4
Units of antithromboplastin activity per ml unknown blood 1 96
The unknown has 140 per cent of the activity of the normal

Sources of Error (a) Poor venepuncture, admixture of tissue juices (b) Failure to add rapidly sample to methanol and to swirl flask as blood is added, thereby getting "caked" blood (c) Failure to remove entirely the ether soluble layer or to separate adequately the aqueous from the ether soluble phase (d) Failure to have an adequately large volume for the supersonic vibrator (Minimal amount that should be used = 5 ml)

Preparation and Assay of Plasma Antithromboplastin Collection of sample The sample of blood is obtained by means of the technic described in detail elsewhere Siliconized glassware is used throughout and the blood is centrifuged in a refrigerated centrifuge at 120 000 total g (g per minute × no of mins) The upper ⅘ of the plasma layer is carefully separated and stored in silicone coated stoppered tube

Preparation of plasma sample Visking casing (1″ in diameter) is cut into approximately 14 inch lengths The lower four inches is moistened under tap water until it becomes sufficiently pliable and then three tight knots are tied in this using the casing material for the tie To the open end of the sac is added a measured amount of plasma to be extracted The portion of the sac projecting above the plasma meniscus is folded over and tied The sac is then suspended vertically from a crossbar or hammock fashion between two stands A revolving fan is placed facing the suspended plasma In this manner a 10 ml sample of normal plasma may be dried in five to six hours

Extraction of the Plasma The sac containing the dried plasma is removed from the crossbar and the material distal to the two knots is cut off and discarded The remainder using sharp scissors, is then cut into small pieces (approximately 3 x 5 mm) These are transferred to a mortar and to them is added 50 to 75 ml absolute methyl alcohol The mixture is then macerated with a pestle by means of both a rotary and a tamping motion, for 7 to 10 minutes The fluid is removed to an Erlenmeyer flask by decantation and a fresh batch of absolute methanol added The above process is repeated until a volume of methanol corresponding to approximately 30 times the initial plasma volume has been added Following this approximately 10 additional volumes are employed in transferring the solid material to the Erlenmeyer flask, rinsing the mortar and pestle etc Finally a total of forty volumes of absolute methanol will have been added for each volume of liquid plasma The residue now contains small discreet well dispersed particles which lend themselves well to extraction This is

allowed to remain at 5°C for five days The details of filtration and distilla tion of the extract and processing obtaining and homogenizing the residue are as described for the tissue lipid antithromboplastin In assaying the activity of the extract allowance must be made for any citrate present by finding the $CaCl_2$ concentration which gives the optimal clotting time in the activated clotting mixture A plasma sample obtained from a normal subject is extracted processed and assayed side by side with the unknown sample.

Results are calculated as described in Chapter XIII, section 3 and expressed either in units of antithromboplastin activity per ml of plasma or in terms of per cent of the normal.

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7 Measurement of Plasma and Serum Antithrombin Activity

J F JOHNSON and W H SEEGERS

There are at least four known natural mechanisms for the inactivation or modification of thrombin action There may be more but these have been demonstrated or can be measured They are (1) The adsorption of thrombin on fibrin Although the amount adsorbed in whole blood or plasma is probably not great this effect is marked in purified systems (2) Heparin co factor present in plasma interfering with the action of thrombin and fibrinogen and thus acting as an antithrombin though probably not a destroyer of thrombin in the strict sense (3) A substance in plasma and serum that inactivates thrombin serving to remove it from

the clotting system (4) A property of both plasma and serum that is active in the removal of thrombin and requires the activation of prothrombin to manifest itself

In considering these effects it has been found convenient to refer to them respectively as antithrombin I, antithrombin II, antithrombin III and antithrombin IV The third action is also known as the natural antithrombin and is the one with which we are concerned in this explanation of the measurement of its strength For measurement of antithrombin II (heparin cofactor, proantithrombin) see page 205

Materials and Reagents (a) *Thrombin* To reduce to a minimum extraneous protein interactions which might lead to faulty results only the purest thrombin available is used as a substrate This is prepared from purified prothrombin in the manner described in another section (page 112) It is possible to use commercial thrombin that has been carefully and repeatedly checked for its activity Thrombin may possibly change its activity when stored at refrigerator temperatures, even becoming more active overnight A constant check is necessary, particularly when commercial products are used in these analytical procedures

Thrombin in a 50 per cent glycerol solution is kept in the deep freeze and in such a solvent it will remain stable for several months It is made up to a strength of at least 1,200 units per ml and preferably more This is buffered with 5 per cent imidazole buffer at pH 7 2 to 7 4 in a concentration of 12.5 per cent by volume of the solution Thrombin in weaker solutions of glycerol, such as 25 per cent is also stable but will freeze when placed in the deep freeze and the thawing process may reduce the strength Refrigerator temperatures will maintain thrombin in glycerol solutions but not to the extent that the deep freeze will

(b) *Plasma* Plasma to be used is collected as described for all the other plasmas a two syringe technique with the usual oxalate or citrate anticoagulants This plasma can be handled with more freedom than those in which Ac globulin or prothrombin are to be measured because the antithrombin is more stable but the usual care will be rewarded by more accurate work and it is advised to maintain the same careful standards for all Antithrombin will withstand several freezings and thawings as well as standing at room temperature for sometime It will remain at the same level in plasma for a few months in the deep freeze and seems to be one of the more stable clotting components When it is desired to do antithrombin studies the plasma must be defibrinated to remove the effect (antithrombin I) of the adsorption of thrombin on fibrin Also thrombin is to be added to this plasma and if there were any fibrinogen present a clot would form that would invalidate the test In order to accomplish this heat is used to defibrinate, the plasma being carefully heated at 56°C for 3 minutes The heat coagulated fibrinogen can then be centri

fuged to the bottom and the supernatant plasma pipetted off for use Light centrifugation is all that is necessary, 1 000 g for 5 minutes will be sufficient This heating does not interfere with the antithrombin effect although higher temperatures may If heating is not desired the plasma may be defibrinated by the addition of a small amount of thrombin as is done for the defibrination of plasma for a prothrombin determination Apparently the addition of 0 1 ml of a solution containing 100 units per ml thrombin to 0 5 ml of plasma will not materially affect the antithrombin determination A comparison of analyses after the two methods of defibrination will reveal the same values

(c) *Serum* This is gathered as described in the accompanying directions for the reagents Since this serum should have no fibrinogen it is not necessary to defibrinate it A slight amount of hemolysis does no harm

Glassware In order to maintain the strength of all thrombin solutions used in the manipulations it is necessary to use coated glassware This may be done with paraffin or silicone Any of the commercial products applied according to the manufacturers directions seems suitable Thrombin is adsorbed on glass and this is an important variable when dealing with the weaker solutions When stronger solutions such as 1 200 to 1 400 units per ml, are handled the coating may be omitted since the adsorbing quality of the glass will be exhausted quickly by these powerful solutions and the percent adsorbed is low

Nevertheless when these analyses are performed coated glassware is used throughout to eliminate this action Re use of siliconized glassware after washing is suspect and is not advisable Paraffin lined tubes can be prepared easily and are satisfactory for repeat use Any break in the continuity of the lining can be seen The determination of the final clotting endpoint does not require coated tubes

Steps in the Procedure This consists of allowing the specimen to react with a measured amount of thrombin for a set period of time and at the conclusion of this time measuring the number of units of thrombin remaining The plasma is first heat defibrinated as described before One ml is commonly heated and then 0 5 ml of this is pipetted into a siliconized or paraffin lined tube after the fibrinogen has been spun down An equivalent volume of thrombin is added to the tube and the two are allowed to remain for two hours at room temperature At the end of this time the tubes are placed in an ice bath to slow the reaction until the amount of thrombin remaining is determined by means of the routine thrombin determination

Therefore where

T = standard concentration of thrombin

t = thrombin concentration in reaction mixture at the final equilibrium

then (a) = thrombin units destroyed by 1 ml of plasma = (T) − 2(t)

and (b) = percentage of thrombin destroyed per ml of oxalated plasma

$$= \frac{(T) - 2(t) \times 100}{T}$$

The answer is expressed as the percentage figure determined by the last equation For the assay of thrombin the routine thrombin analysis described by Seegers and Smith is followed This consists of the dilution of the mixture until a standard solution of fibrinogen is clotted in 15 seconds by the amount of thrombin contained therein (see page 120)

Precautions The selection of the two hour time period for inactivation of thrombin is based on the fact that in the first 60 minutes of the reaction the destruction of the thrombin is progressive becoming gradually less until an equilibrium is reached between the formation and destruction of the thrombin This equilibrium has been observed in a large number of plasmas and always has been reached before two hours Under these conditions of balance the thrombin will be stable for a long time

The amount of thrombin in the standard solution is varied with the different specimens to be tested, a smaller amount for the smaller amount of antithrombin believed to be present, and the reverse for the stronger samples If human plasmas are to be used routinely a standard solution of about 1 400 units per ml can be stored and used with satisfactory results These relationships between amounts of material used and inactivation are shown in table 1

With each group of specimens a blank is run at the same time using saline instead of plasma to mix with the thrombin At the end of the two hours there should be no destruction of thrombin in this system, and it can be used to check the test In addition to the blank run with the specimen, a normal plasma of the same species should be examined concurrently

TABLE 1 *Comparison of Potential Thrombin Inactivating Capacity of Serum and Defibrinated Plasma*

Units Thrombin in Substrate	Units Thrombin destroyed by 1 cc		Difference
	Plasma	Serum	
200	195	130	65
300	280	165	115
500	400	220	180
800	455	220	235
1100	375	165	210
1450	250	115	135

This additional determination serves as a control on the activity of the reagents

Serum When serum is used the procedure is the same It can be seen from table 1 that there is little less antithrombin activity in serum than in plasma Of course as stated before there is no defibrination with serum and the process is thus simplified by the omission of this step

In either of the analyses on serum or plasma it is advisable to run at least three determinations of the end point These should check within a fraction of a second of each other to be sure that an equilibrium has been established both as regards the antithrombin thrombin system and also the dilution of the thrombin in the analysis

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8 Estimation of Antithrombin in Plasma (Short Incubation Method)

E C LOOMIS

Object of the Test To determine the amount of active antithrombin present in plasma or serum

Principle Underlying the Test Antithrombin reacts with thrombin neutralizing or blocking its ability to act on fibrinogen This principle may be utilized in the assay by supplying an excess of thrombin and determining the amount left after a standard time interval of incubation

Reagents and Apparatus Required (1) Plasma for assay (2) Oxalated saline 0 075 per cent $K_2C_2O_4$ in 0 85 per cent sodium chloride (3) Purified bovine thrombin (Thrombin Topical Parke Davis & Co) (4) Purified bovine fibrinogen[1] (approximately 0.3 per cent clottable protein in oxalated saline buffered with imidazole[2] (5) Antithrombin titration mixture 7 parts oxalated saline 1 part imidazole buffer 3 parts 15 per cent purified

acacia (calcium free) in saline (6) 10 × 75 mm test tubes (7) Pipets serologic (8) Stop watch

Steps in Performance of the Test (a) Prepare an 800–1000 unit per ml thrombin solution in oxalated saline Assay the solution by the two stage technique (b) Add 0 4 ml plasma or heat-defibrinated plasma[3] to 1 ml of the thrombin solution Allow to react for five minutes (c) Assay the remaining thrombin by the two-stage technic

Calculation Units of antithrombin per ml = 2 5 (A–C) A = Dilution × 5 × 1 5 × 1 = units of thrombin in original solution C = Dilution × 5 × 1 5 × 1 4 = units of thrombin remaining

Normal Range of Values The range of values obtained in normal human plasma is from 200 to 500 units per ml As more determinations are recorded the normal range may be narrowed

Precautions and Sources of Error (1) Contamination of reagents with antithrombin (2) If the determination is made on whole plasma the clotted fibrin must be wound on a glass rod but not removed from the reaction mixture However, the fibrin will adsorb thrombin and give an apparently higher antithrombin titer (3) Any contamination with heparin will also increase the apparent antithrombin since it activates proantithrombin (see page 205) (4) For greatest accuracy and reproducibility of results, about half of the thrombin should be neutralized by the antithrombin If the deviation is beyond the range of 40 to 60 per cent the determination should be repeated using either a dilution of the plasma or a different initial thrombin concentration

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9 Estimation of Antithrombin Activity (Rapid Method of Wilson)

Adapted by R R HOLBURN*

Principle The thrombin in normal blood which is allowed to clot spontaneously, is almost entirely inactivated in two hours, indicating the presence of an antithrombin By adding a measured amount of thrombin to defibrinated plasma or serum the loss in thrombin activity after a stated period of incubation provides a measure of the antithrombin activity in those fluids

Apparatus and Reagents Plasma or serum Venous blood is obtained and allowed to clot or is mixed with one-seventh of its volume of a 1 85 per cent solution of potassium oxalate After centrifugation, the hematocrit reading and total volume of the oxalated blood is recorded to permit correction for dilution by the oxalate The plasma or serum is incubated in a waterbath for 10 minutes at 56 C The precipitated fibrinogen is then removed from the plasma by centrifugation

Fibrinogen Prothrombin free fibrinogen is prepared by the method of Warner, Brinkhous and Smith (see page 107) and stored at −35 C

Thrombin Thrombin is prepared according to the method of Seegers Brinkhous Smith and Warner (see page 120) and standardized either by the dropper or pipet method

Steps in Procedure Standardization of thrombin solution 1.5 ml of 0 85 per cent NaCl solution is added to each of 9 test tubes A series of 9 dilutions containing 0 2 to 1 0 unit of thrombin in each 0 1 ml is prepared and aliquots of 0 1 ml are added in sequence to the tubes of saline To each test tube is added 0 5 ml of the fibrinogen solution (a total of 2 1 ml) The end point is the appearance of fibrin strands timed with a stopwatch A graph is plotted of the values obtained to be used for the quantitative determination of antithrombin activity

Antithrombic activity of plasma and serum Dilutions of the plasma or serum of 1 40 1 50 and 1 60 are made with 0 85 per cent NaCl solution To 1 5 ml of the plasma dilution is added 0 1 ml of thrombin solution containing 1 unit of thrombin The mixture is then allowed to incubate for exactly 4 minutes at 28 C before the addition of 0 5 ml of fibrinogen The end point is the same as in the standardization test For accurate readings the clotting times between 28 and 35 seconds should be used to determine the dilution to be used in the calculation

* From Archives of Internal Medicine 69 647 1942

Calculation On the basis of observed quantitative activity, 1 unit of antithrombin is defined as that amount which will inactivate 1 unit of thrombin in 4 minutes at 28°C In calculating the antithrombic unitage in plasma, correction is made for the oxalate dilution factor No correction is necessary when serum is being tested The denominator of the final dilution is then multiplied by the amount of thrombin inactivated and the result multiplied by the factor 5 28 (the correction value necessary to correlate the values of diluted and undiluted specimens, as determined by the author) The activity is expressed in units of antithrombin per ml of serum or plasma

Values Obtained Human subjects dogs, cats and cattle average 90 units/ml Guinea pigs, hogs and rabbits slightly higher values Albino rats 123 units/ml (average) There is little if any, difference in the antithrombic activity of plasma and serum

Precautions and Sources of Error No variations occur in the antithrombic unitages between temperatures of 25 to 37°C providing the thrombin is standardized at the temperature utilized in the ultimate test During conversion of fibrinogen to fibrin about 5 1 units of thrombin disappear through adsorption Apparently the saturation point of the adsorption of thrombin on fibrin is quickly reached when the conversion occurs in the presence of excess amounts of thrombin Since plasma contains 2 2 to 4 5 mg fibrinogen per ml in a plasma containing 300 units of potential thrombin only 11 0 to 22 5 units are adsorbed during the conversion of fibrinogen to fibrin

The ability of serum or plasma to inactivate thrombin is destroyed by incubation for 10 minutes at 66 to 67 C The step of heating the plasma must be done cautiously

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10 Estimation of Proantithrombin (Heparin Cofactor) in Plasma

E C LOOMIS

Object of the Test To differentiate between proantithrombin and antithrombin in plasma to determine the reserve capacity for thrombin neutralization

Principle Underlying the Test Proantithrombin is activated to anti thrombin by heparin By determining the antithrombin by the method on page 201 and the total activated proantithrombin and antithrombin the quantity of proantithrombin may be calculated

Reagents and apparatus required (1) Plasma for assay (2) Oxalated saline 0 075 per cent $K_2C_2O_4$ in 0 85 per cent sodium chloride (to buffer add 4 ml imidazole to 96 ml saline) (3) Purified bovine thrombin (Thrombin Topical Parke Davis & Co) (4) Purified bovine fibrinogen [1] (Approximately 0 3 per cent clottable protein in oxalated saline buffered with imidazole [2] (5) Heparin 2 units per ml in oxalated saline (6) Antithrombin titration mixture 7 parts oxalated saline 1 part imidazole buffer 3 parts 15 per cent purified acacia (calcium free) in saline (7) Pipets serologic (8) 10 x 75 mm test tubes (9) Stop watch

Steps in the Performance of the Test (a) Add dry powdered thrombin to the heparin solution until the solution assays 800–1000 units of thrombin per ml Assay this solution by the two stage thrombin procedure (b) Add 0 4 ml plasma to 1 ml of the thrombin solution (Do not use heat treated plasma) Allow to react for five minutes (c) Assay the remaining thrombin by the two stage technic (d) Assay the plasma antithrombin by the procedure on page 201

Calculation U of proantithrombin per ml = total from this determination − result from antithrombin determination Total antithrombin in u /ml = 2 5 (A–C) A = Dilution × 5 × 1 5 × 1 = units of thrombin in original solution C = Dilution × 5 × 1 5 × 1 4 = units of thrombin remaining

Normal Range of Values Human plasma contains 400 to 800 units of proantithrombin per ml The total available antithrombin and proantithrombin will therefore be as high as 1200 to 1400 units per ml

Precautions and Sources of Error (1) Contamination of reagents with antithrombin (2) Whole plasma must be used in this determination because heating at 56 C for 3 to 5 minutes will destroy a variable amount of proantithrombin When the fibrin clot is wound on a glass rod to free the serum thrombin solution for the thrombin assay the fibrin should

remain in the solution Results will be more constant and reproducible because of the more uniform error of thrombin adsorption on the fibrin (3) For greatest accuracy and reproducibility of results about half of the thrombin should be neutralized by the antithrombins If the deviation is beyond the range of 40 to 60 per cent the determination should be re peated, using either a dilution of the plasma or a different initial thrombin concentration (4) It may be seen readily that the determination is not an absolutely quantitative procedure, but relative results are obtainable

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71 Extraction of Heparin from Blood[1]

L B JAQUES

Heparin, while not normally present in the blood, appears in it under certain pathologic conditions (peptone shock, anaphylactic shock) The following method may be used to extract it for assay in such states, and from the blood of subjects who have been receiving the drug

Reagents Sodium citrate 3 8 per cent ether phenol solution Phenol is liquified in a water bath and 800 ml are diluted to one liter with distilled water

Procedure Nine ml of blood are drawn and added to 1 ml of sodium citrate solution and centrifuged at 2,000 r p m for ten minutes The plasma is pipetted off and the cells are washed with 1 ml of 0 85 per cent NaCl This wash is added to the plasma and the cells are discarded Phenol solution, 5 5 ml is added the tube is corked and the phenol and plasma are thoroughly mixed by vigorous shaking The tube is allowed to stand at room temperature for ten to twelve hours after which it is centrifuged at 2 500 rpm for 20 minutes The clear upper layer is pipetted off carefully and the phenol layer washed with a small amount of 0 85 per cent NaCl The combined aqueous phase is washed with 5 ml of ether The ether layer is pipetted off and the ether remaining in solution is removed by vacuum or by heating in a water bath for a minute or two at 65°C The

solution may be then assayed by the methods described on page 209 For routine or clinical work the method can be shortened by omitting the two washings It has been found that even with this latter change highly reproducible recoveries (80 per cent) can be obtained by this procedure For smaller or larger volumes of blood, quantities of saline phenol, etc are taken in the given proportion An earlier alternative method is described by Jaques Monkhouse and Stewart[2]

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CHAPTER XIII

ANTICOAGULANTS OF TISSUE ORIGIN

1 Heparin Methods of Assay

L B JAQUES

Principles Heparin may be detected by direct or indirect methods Indirect methods are (1) clotting time determinations and (2) protamine (toluidine blue) titration Direct methods involve the extraction of the heparin by (1) the Charles and Scott procedure for tissues or (2) the Monkhouse and Jaques procedure for blood, followed by identification and measurement of the heparin obtained Two problems are involved in such measurements—identification and quantitative determination These are apt to be confused The only satisfactory means of identifying heparin is isolation followed by observation of a number of properties crystalline form, solubility sulphur content optical rotation specific biologic activity specific metachromatic activity etc No one property is unique to heparin but a combination of these measurements gives a dependable result It should be appreciated that impurities in the extract can seriously interfere with measurements either directly as when the presence of thromboplastic activity causes a low reading in a clotting test or indirectly as when the presence of amino acids interfere with metachromatic readings All tests must be conducted by direct comparisons with standard beef heparin Since different species show different values for the ratio of anticoagulant activity (clotting time effect etc) metachromatic activity (protamine titre protein combining power) this ratio is very useful for identification and also for evaluation of the reliability of the data obtained

Use of Clotting Times[1] Where heparin is being injected and it is known that other changes will not occur in the clotting system this is the simplest procedure The technic is discussed on page 26 It is possible by drawing blood in silicone coated syringes and glassware and mixing with known quantities of heparin, to construct a standard curve for converting clotting times to heparin concentrations

Protamine Titration Protamine is a basic protein Heparin is acidic and forms a salt with protamine Both are anticoagulants However on

adding varying quantities of protamine to heparinized blood an amount of protamine can be found which restores the clotting time to normal

Reagents and Equipment 8 mm test tubes rack water bath stop watch 5 ml siliconed syringe with Arquad treated needle Protamine solutions, 1 mg /ml and 0 1 mg /ml in isotonic saline, with pH adjusted to 7 0

Procedure 8 mm test tubes are cleaned by washing in soap and water rinsing thoroughly with distilled water dried and finally cleaned in chromate-concentrated sulphuric acid solution After rinsing and boiling in distilled water they are rinsed and dried The test tubes are placed in a rack in a water bath at 37 C in groups of six The following amounts of protamine are measured into the tubes 0 5 0 1 0 05 0 02 0 005 0 0 mg Volume is made up to 0 5 ml with isotonic saline About 4 cc of blood is taken by vein puncture The needle is removed the half ml discarded and 0 5 ml of blood added to each tube mixed and clotting times measured In addition to reading the 1st tube clotted the second and third are noted as this allows estimation of an end point occurring between tubes For toluidine blue the test is performed similarly but substituting the dye for the protamine

Sources of Error Each lot of protamine must be standardized as the ratio changes with the lot number Protamine has other effects on the clotting system In addition to its inhibitory action and power to neutralize heparin it clumps and destroys platelets It is therefore not specific Also *the quantity of protamine is the quantity required to depress heparin* activity in blood not the amount to combine with heparin In using the test a safe rule is that a clotting time three times the normal value or more if reduced to normal by adding protamine can be attributed to heparin and the titration reported in heparin units Lesser changes should be reported as questionable unless supported by other tests

Biologic Assay Procedures for Heparin [2, 3, 4] As with any therapeutic substance in use the anticoagulant activity of heparin must refer to its activity on the actual biological system itself i e the clotting of whole blood This was the principle adopted by Howell in his original cat assay and also with modifications by Charles and Scott [5] Jorpes used beef blood and Schutz rabbit blood It can be extended for special problems to human blood It would be advisable to limit the term anticoagulant activity to such tests on the clotting of fresh whole blood

Because of inability to obtain whole blood in many laboratories it is usual now for many purposes to measure some other property of heparin Fischer and Schmitz in 1932 used inhibition of clotting of chicken plasma since it is relatively simple to collect chicken blood without any anticoagulant and after separating off the cells preserve the plasma for the assay

This was also used by Dam and Glavind in 1939 Jaques and Charles used the clotting of oxalated blood by thrombin Many have used plasma clotted with calcium and thromboplastin Reinert and Winterstein (1939) and Foster (1942) used ox plasma, Kuizenga, Nelson and Cartland (1943) used sheep plasma MacIntosh (1941) used horse plasma and excess thromboplastin P Astrup (1947) used citrated human plasma, McGoon (1950) used recalcified citrated human blood Astrup and Galmar (1944) who used ox plasma found that with this system, synthetic sulfonated polysaccharides showed much greater activity than heparin although their anticoagulant activity was much less so that some of these tests do not measure anticoagulant activity at all Jalling et al [2] discuss these methods and report discrepancies observed with their use The U S Pharmacopeia[1] has adopted the sheep plasma method of Foster as the official test The British Pharmacopeia gives the cat blood sheep plasma and other methods as alternatives

With all methods which use a clotting system for the assay of heparin a choice has to be made between estimating degree of clotting with varying amounts of heparin at a fixed time and estimating the clotting times with varying amounts of heparin The factors involved in this choice are discussed by Jaques and Charles [4]

It must be appreciated for physiologic and pathologic studies, that many of these methods do not distinguish between heparin and its metabolized or inactive forms, and that many of them also are of doubtful specificity for heparin On both these points the original assay procedures using fresh whole blood while not free from criticism are more reliable than the other methods

Heparin Units Howell originally defined the activity of heparin in terms of units one unit being that amount which prevented the clotting of 1 ml of cat's blood in the cold for 24 hours A number of factors such as temperature are not made clear in this definition and can cause as much as a five fold difference in activity However the unit used by Charles and Scott is as close to the original unit of Howell as can be determined at the present time The international unit which was adopted in 1942 by the League of Nations is of the same order of potency as the Charles and Scott unit and therefore of the original Howell unit [4a] It is the activity of $\frac{1}{130}$ mg of the international standard powder which is prepared in the form of the sodium salt of heparin

All assays must be conducted against a standard preparation of beef heparin assayed carefully by the same procedure against a sample of the international standard heparin powder In the U S A this can be obtained from the U S P Reference Standards, 46 Park Avenue New York 16 N Y and in other countries from the similar authority

Since activity varies with species[7] it is advisable in physiologic and pathologic studies to express heparin concentrations where possible in milligrams This requires the isolation of heparin from the species under study in order to establish its absolute unitage in relation to the beef heparin standard

Howell Method (as Modified by Charles and Scott[4]) of Assay for Heparin

Reagents Carefully selected 8 mm Widal tubes marked at a volume of one ml are used The tubes are grouped according to the bore and only those of one group are used in one set of assays A standard heparin solution containing 0 025 mg /ml (2 5 units/ml) of the crystalline barium salt of beef heparin is used All solutions are made up in 0 85 per cent NaCl solution containing 0 3 per cent of tricresol A cat is anesthetized by intraperitoneal injection of amytal One carotid artery is exposed and a clean glass cannula inserted

Procedure The unknown solution is diluted to approximately the same strength as the standard as indicated by a preliminary assay Four dilutions of the unknown in this range are taken such that there is a difference of 10 per cent between each dilution 0 1 ml 0 2 ml 0 3 ml of each solution (the standard and four dilutions of the unknown) are measured into each group of three tubes and the volume is adjusted to 0 3 ml with 0 85 per cent NaCl solution These are placed in a rack holding 18 tubes The tubes are filled to the 1 ml mark with blood from the cannula All the tubes in the rack containing only 0 1 ml of the solutions are filled first then those with 0 2 ml and then those with 0 3 ml The standard solution is taken first then strongest dilutions of unknown followed by other dilutions in order As each tube is filled it is mixed by inverting twice in such a manner that the blood covers the inside area of the tube Three such racks may be filled at the same time but each must have its own standard and be filled separately The racks are placed in a water bath at 25 C for two hours and the tubes are covered to prevent evaporation Since with an occasional cat all the tubes will be clotted after two hours it is advisable to inspect the tubes after an hour and a half and if all the tubes of the weakest dilution appear to be almost completely clotted to read the tubes then such a condition is readily seen since settling of the red cells with the resultant buffy coat can normally be observed whereas if the blood is clotting too rapidly this does not occur The tubes are read by tipping each tube and judging the degree of clotting A complete clot which does not break upon tipping is recorded as + whereas completely fluid blood is recorded as − Intermediate stages representing decreasing degrees of coagulation may be designated by D+ D dd d At least four intermediate stages can be distinguished and with practice eight can be

This was also used by Dam and Glavind in 1939 Jaques and Charles used the clotting of oxalated blood by thrombin Many have used plasma clotted with calcium and thromboplastin Reinert and Winterstein (1939) and Foster (1942) used ox plasma, Kizenga Nelson and Cartland (1943) used sheep plasma MacIntosh (1941) used horse plasma and excess thromboplastin P Astrup (1947) used citrated human plasma, McGoon (1950) used recalcified citrated human blood Astrup and Galsmar (1944) who used ox plasma found that with this system, synthetic sulfonated polysaccharides showed much greater activity than heparin, although their anticoagulant activity was much less so that some of these tests do not measure anticoagulant activity at all Jalling et al [2] discuss these methods and report discrepancies observed with their use The U S Pharmacopeia[6] has adopted the sheep plasma method of Foster as the official test The British Pharmacopeia gives the cat blood sheep plasma and other methods as alternatives

With all methods which use a clotting system for the assay of heparin a choice has to be made between estimating degree of clotting with varying amounts of heparin at a fixed time and estimating the clotting times with varying amounts of heparin The factors involved in this choice are discussed by Jaques and Charles [4]

It must be appreciated for physiologic and pathologic studies that many of these methods do not distinguish between heparin and its metabolized or inactive forms and that many of them also are of doubtful specificity for heparin On both these points the original assay procedures using fresh whole blood while not free from criticism are more reliable than the other methods

Heparin Units Howell originally defined the activity of heparin in terms of units one unit being that amount which prevented the clotting of 1 ml of cat s blood in the cold for 24 hours A number of factors such as temperature are not made clear in this definition and can cause as much as a five fold difference in activity However the unit used by Charles and Scott is as close to the original unit of Howell as can be determined at the present time The international unit which was adopted in 1942 by the League of Nations is of the same order of potency as the Charles and Scott unit and therefore of the original Howell unit [6a] It is the activity of $\frac{1}{130}$ mg of the international standard powder which is prepared in the form of the sodium salt of heparin

All assays must be conducted against a standard preparation of beef heparin assayed carefully by the same procedure against a sample of the international standard heparin powder In the U S A this can be obtained from the U S P Reference Standards 46 Park Avenue New York 16 N Y and in other countries from the similar authority

in paraffined vessels, and the plasma diluted with an equal volume of Ringer s solution The thromboplastin is prepared from chicken breast muscle following the specifications of Schonheyder [4]

Procedure In the assay 0 25 ml of diluted plasma +0 05 ml of heparin solution + 0 05 ml of thromboplastin are mixed in a 8 mm test tube and placed in the Fischer test tube rack in a water bath at 37 C The tube is examined by tilting at 10- econd intervals A dilution of thromboplastin is taken such that a clotting time of 30 to 90 sec is obtained with the plasma plus Ringer s solution For the assay a series of tubes containing various dilutions of unknown and standard heparin in Ringer s solution are prepared including a tube without heparin and the clotting times deter mined It is not clear from Fischer s description whether he used a set of tubes of the standard heparin solution each time or not This is a first essential for the assay, since Astrup and Astrup found that the heparin activity of a solution as measured by the value of K (see further on) varied considerably with different plasmas and our data indicate that it changes during a day s assays Hence it is necessary always to compare the potency of the unknown with that of the standard setup at the same time Four dilutions of the standard are made up such as to give measureable clotting times and also four dilutions of the unknown of approximately the same potency as the dilutions of the standard as determined by preliminary assay 0 05 ml of each of these is added to the plasma in eight clotting tubes and to a ninth tube Ringer s solution is added To avoid any bias due to the order of mixing the order of the tubes is changed in each assay before adding the thromboplastin In order to initiate clotting simultane ously in all the tubes the empty tubes are placed in a bath of ice and water The plasma heparin and thromboplastin are added in this order and mixed while still in the ice bath The rack is then transferred to the bath at 37 C and the clotting time reckoned from the time of immersion in the 37 C bath This procedure gives consistent results and shows a linear relation between heparin concentration and log of clotting time as required by Fischer and Astrup

Fischer reports the activity of heparin by its K value the slope of the line obtained by plotting log clotting time against heparin concentration K is found by the method of least squares the clotting time with zero heparin being included The heparin activity of the unknown is then calcu lated from the ratio K (unknown) K (standard) Typical values of K and K are 3 95 and 3 97 4 24 and 4.34 4 39 and 4 61 These were three assays of a standard solution conducted on the same plasma in the course of eight hours It can be seen that the K value changes as the plasma ages The potency of the unknown in the three assays was 99 5 97 7 95.2 (theoretic —100 0)

detected After completing the reading, the data is examined, to compare the standard with the unknown In a good assay the readings for the tubes containing the standard should match with those for one dilution of the unknown The ratio of the potency of the unknown is to that of the standard as the respective dilutions of the two Where the readings of the standard lie between those of two of the dilutions, it is possible to estimate the potency of the unknown but where they lie beyond those of any of the dilutions of the unknown it is necessary to make further assays using new dilutions of the unknown

The Thrombin Method of Assay for Heparin[4]

Reagents Citrated beef or dog blood Thrombin 1 ampoule (5000 units) of ' Topical Thrombin ' dissolved in one half volume of 0 85 per cent NaCl and made up to volume with 50 per cent glycerol This is kept in refrigerator and diluted with 0 85 per cent NaCl as needed 8 mm wide (i d) test-tubes Water bath at 25°C Standard heparin 0 1 units per ml prepared from reference standard

Procedure The thrombin solution is standardized by adding varying amounts to a clotting system composed of 0 5 ml of blood, 0 2 and 0 3 ml of standard heparin and 0 85 per cent NaCl solution to give a total volume of 1 0 ml , and examining the tubes after standing fifteen minutes at 25°C That amount of thrombin which is just sufficient to cause clotting with 0 2 ml of heparin and not with 0.3 ml is then taken for the assay The stock thrombin solution is diluted as required to contain this amount of thrombin in 0 1 ml For the assay, 0 20 0 22, 0 25 0 27, 0 30 ml of the heparin standard are taken in carefully selected tubes (as described for the Howell method) The volume is made up to 0 4 ml with 0 85 per cent NaCl and 0 50 ml of oxalated beef blood is added In another series of tubes, equivalent amounts of the unknown, as found by preliminary assay are taken The tubes are allowed to stand in a water bath at 25°C for exactly ten minutes to come to temperature equilibrium and then 0 1 ml of the thrombin solution is added to each tube the system being mixed immediately by inverting the tube twice The tubes are allowed to stand in a constant temperature water bath at 25°C for fifteen minutes and then read Below a certain concentration of heparin the clot is found to be solid and slides down the tubes Above this concentration which is the end point the clot breaks up and with higher concentrations of course no clot is formed The end point will be found between one pair of tubes

Fischer Assay[4] *for Heparin*

Reagents Chicken blood is collected by paraffined syringe from a wing vein or by paraffined cannula from the carotid artery, chilled centrifuged

in paraffined vessels and the plasma diluted with an equal volume of Ringer s solution The thromboplastin is prepared from chicken breast muscle following the specifications of Schonheyder [4]

Procedure In the assay 0 25 ml of diluted plasma +0 05 ml of heparin solution + 0 05 ml of thromboplastin are mixed in a 8 mm test tube and placed in the Fischer test tube rack in a water bath at 37 C The tube is examined by tilting at 10 second intervals A dilution of thromboplastin is taken such that a clotting time of 30 to 90 sec is obtained with the plasma plus Ringer s solution For the assay a series of tubes containing various dilutions of unknown and standard heparin in Ringer s solution are prepared including a tube without heparin and the clotting times determined It is not clear from Fischer s description whether he used a set of tubes of the standard heparin solution each time or not This is a first essential for the assay since Astrup and Astrup found that the heparin activity of a solution as measured by the value of K (see further on) varied considerably with different plasmas and our data indicate that it changes during a day s assays Hence it is necessary always to compare the potency of the unknown with that of the standard setup at the same time Four dilutions of the standard are made up such as to give measureable clotting times and also four dilutions of the unknown of approximately the same potency as the dilutions of the standard as determined by preliminary assay 0 05 ml of each of these is added to the plasma in eight clotting tubes and to a ninth tube Ringer s solution is added To avoid any bias due to the order of mixing the order of the tubes is changed in each assay before adding the thromboplastin In order to initiate clotting simultaneously in all the tubes the empty tubes are placed in a bath of ice and water The plasma heparin and thromboplastin are added in this order and mixed while still in the ice bath The rack is then transferred to the bath at 37 C and the clotting time reckoned from the time of immersion in the 37 C bath This procedure gives consistent results and shows a linear relation between heparin concentration and log of clotting time as required by Fischer and Astrup

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Fischer Assay[5] *for Heparin*

Reagents Chicken blood is collected by paraffined syringe from a wing vein or by paraffined cannula from the carotid artery, chilled centrifuged

each tube and mix the contents by inverting three times in such a way that the entire inner surface of the tube is wet

In the same manner use the test dilution of the preparation under assay in a series completing the entire process of preparing and mixing the tubes of the Standard and of the preparation under assay respectively within 20 minutes Exactly one hour after the addition of the calcium chloride determine the extent of clotting in each tube recognizing three grades (0 25 0 50 and 0 75) between zero and full clotting (1 0) If no tube of a series is graded more than 0 5 or if no tube is graded less than 0 5 repeat the assay using appropriately modified test dilutions

Calculation of the Potency Note or ascertain by interpolation the respective volumes of the test dilutions of the Standard and of the preparation under assay required for the 0 5 grade of clotting From these volumes determine the weight in mg of U.S P Heparin Sodium Reference Standard to which 1 mg of the heparin sodium is equivalent in activity

Chemical Assays[8, 9]

The multiplicity of reactions given by heparin with complex bases and proteins have made possible the development of a great number of assay procedures based on physical and chemical tests Fischer and Schmitz devised an assay based on the effect of heparin on turbidity of casein This still appears to be as satisfactory an assay as any developed later Various alternatives of this procedure have been developed MacIntosh for example used the amount of toluidine blue remaining in solution after precipitation by heparin This has also been used by Copley and Whitney (1944) and Trethewie and Melvin (1945) while Gibson[9] has measured the amount of dye precipitated by heparin Winterstein has recently developed a dibenzofuran derivative (No 1261) which gives stable suspensions with heparin and hence turbidity measurements can be used

While the metachromatic properties of heparin are rather unique the only quantitative method using it as a basis for assay is that described by Jaques Mitford and MacDonald and Jaques Monkhouse and Stewart[10] Copley described a crude semiquantitative method using this principle As a qualitative test for identification of heparin it has been used very successfully by Scandinavian workers in many investigations

Metachromasia refers to a shift in the absorption spectrum of a dye in the presence of certain complexes Heparin and related acid polysaccharides show this property to a high degree and in the presence of sufficient sodium chloride which depresses the activity of hyaluronic acid and similar substances the method is fairly specific for the heparin series of compounds It should be emphasized that the term metachromatic activity can only be applied if the shift in absorption spectrum is measured

*U.S P Assay**

Reagents Standard solution of heparin Weigh accurately about 50 mg of U S P Heparin Sodium Reference Standard previously dried at 60 C over phosphorous pentoxide to constant weight, and dissolve in sufficient 0 85 per cent NaCl solution to yield a concentration of 1 mg /ml Store in a tightly stoppered vessel in a cool place and use it for no longer than 3 months after preparation

Preparation of plasma Collect blood from sheep directly into a vessel containing 8 per cent sodium citrate solution in the proportion of one volume to each 19 volumes of blood to be collected Mix immediately by gentle agitation and inversion of the vessel Promptly centrifuge the blood and pool the separated plasma To a 1 ml portion of the pooled plasma in a clean test tube, add 0 2 ml 1 per cent calcium chloride solution and mix Consider the plasma suitable for use if a solid clot forms within 5 minutes To store plasma for future use subdivide the pooled lot into portions not exceeding 100 ml in volume freeze the portions at −20°C or below and store at a temperature not exceeding −8 For use in the assay thaw the frozen plasma in a water bath not exceeding 37°C Remove particulate matter by straining the thawed plasma through a coarse filter

Procedure Weigh accurately about 25 mg of heparin sodium, previously dried at 60 C over phosphorous pentoxide to constant weight in sufficient 0 85 per cent sodium chloride solution to give a concentration of 1 mg per ml

Determine by preliminary trial if necessary approximately the minimum quantity of *standard solution of heparin* which when added in 0 8 ml of 0 85 per cent NaCl solution maintains fluidity in 1 ml of prepared plasma for one hour after the addition of 0 2 ml of one per cent calcium chloride solution This quantity is usually between 01 and 015 ml of the Standard solution On the day of the assay prepare a test dilution of the Standard solution such that it contains in each 0 8 ml of 0 85 per cent NaCl solution the above determined quantity of the Standard solution

In the same manner dilute the solution of heparin sodium prepared as directed above

Clean hard glass 13 x 100 mm test tubes by immersion overnight in chromic acid cleansing mixture (page 628 U S P XIII) Fit the tubes with paraffin coated corks To a series of these tubes add graded amounts of the test dilution of the Standard, selecting the amounts so that none exceeds 0 8 ml and so that they correspond roughly to a geometric series in which each step is approximately 5 per cent greater than the next lower To each tube so prepared add 1 0 ml of prepared plasma and sufficient 0 85 per cent NaCl solution to make the total volume 1 8 ml Add 0 2 ml of 1 per cent calcium chloride solution note the time immediately stopper

have a high dispersion, allowing the use of a small spectral band witdth The Coleman Jr Spectrophotometer with absorption at 510 mμ is satis factory also the Beckman DU spectrophotometer with the photocell replaced by a Photovolt photomultiplier attachment (cf H B Collier R P Barschel Analytic Chemistry vol *24*, page 1030, June 19o2) With the last the dye buffer and 0 85 per cent NaCl are used as a water blank giving a greatly increased sensitivity

Sources of Error These may be due to the dye the sample or the meas uring instrument While the reaction was first described for toluidine blue, authentic samples of this dye do not give the reaction The reaction is due to Azure A However dye samples do change with time The stock solution must be kept in the cold and renewed monthly or even earlier, whenever 80 per cent of the stock has been used up Only certified dye can be used Many impurities in crude heparin preparations interfere with the reaction If a photoelectric colorimeter is used the absorption spectra for dye and dye + heparin must be run with the instrument to determine the position of the absorption band in the particular instrument and whether the in strument will detect it with sufficient sensitivity Finally it should be remembered that the absorption band can be made to disappear by heat and by alcohol and that the pH of the mixture should be checked when dealing with many solutions

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and if the pH of the reaction is below 10 The dyes Azure A and toluidine blue have been used as (1) precipitating agents for heparin (2) antidotes for heparin (3) metachromatic agents Their use as (1) has been discussed elsewhere, (2) is the same as the protamine titration for heparin (see page 209) Azure A and toluidine blue have an absorption maximum at 620 mμ On the addition of heparin this is depressed and a new band appears at 500 mμ The change in these bands is a function of the heparin concentration but at 550 mμ, the presence of heparin does not affect absorption These dyes are also acid base indicators above pH 10 giving a red color with alkali Hence, measurements in alkaline solutions are not measurements of metachromasia As examples of these methods, the turbidity method of Winterstein and the metachromatic method of Jaques et al are described As already indicated, these methods do not readily distinguish between heparin, its precursors and metabolic products, and require checking by other methods

Turbidity Method of Marbet and Winterstein[11]

Reagents Four per cent solution of 2 Dimethylamino-methyl-dibenzofuran hydrochloride (Roche) Ten per cent hydrochloric acid Kieselgur, in case of urine containing protein Nephelometer

Procedure Four ml sample is placed in nephelometer tube one drop of 10 per cent HCl added and reagent added to the 8 ml mark The contents are mixed the tube allowed to stand 5 minutes and the turbidity read in a nephelometer Marbet and Winterstein describe a paper nephelometer graduated in mg per cent heparin obtainable from F G Hoffmann La Roche & Company A G, Basel While developed for urine, the method can be used for other protein free heparin solutions

Metachromatic Assay of Heparin[10]

Reagents 100 mg per cent Azure A (certified biologic stain) Diluted 1 8 5 before use 0 15 M potassium phosphate buffer pH 7 3 0 85 per cent NaCl

Procedure The unknown heparin solution is measured into the colorimeter cell and the volume made up to 2 0 ml with the NaCl solution Two ml of buffer is added and 1 0 ml of the Azure A solution, mixed and the light absorption measured at 500 mμ The heparin concentration is determined from a standard curve constructed with known quantities of heparin

The selection of a suitable colorimeter is important The Lovibond tintometer has proved satisfactory With this the complementary color of the absorption band is matched visually against standard glasses to give a curve Lovibond red units vs heparin Photoelectric colorimeters must

have a high dispersion allowing the use of a small spectral band width The Coleman Jr Spectrophotometer with absorption at 510 mμ is satisfactory, also the Beckman DU spectrophotometer with the photocell replaced by a Photovolt photomultiplier attachment (cf H B Collier, R P Barschel Analytic Chemistry, vol *24* page 1030 June 1952) With the last the dye buffer and 0 85 per cent NaCl are used as a water blank giving a greatly increased sensitivity

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2 Heparin Preparation and Purification

L B JAQUES

The following is the method devised by Charles and Scott[1 2] for extraction of heparin from the tissues

Reagents For each 100 grams of tissue N/2 sodium hydroxide (150 ml), saturated ammonium sulphate (18 ml), 95 per cent ethanol Difco trypsin (200 mg)

Procedure The tissue is minced and allowed to stand for autolysis at 25°C for 24 hours The sodium hydroxide and saturated ammonium sulphate solutions are then added and the mixture heated to 50°C in a water bath, kept at this temperature for 30 minutes, then heated to 70°C The tissue is then filtered immediately through Chain cloth and allowed to drain Large quantities of tissue are strained through cheese cloth or open wire mesh The filtrate is acidified to pH 2 0 to 2 5 with sulphuric acid heated to 65°C and centrifuged The precipitate is washed with hot water containing sufficient sulphuric acid to pH 2 0

The precipitate is then extracted at room temperature for 20 hours with 95 per cent ethyl alcohol The mixture is then centrifuged and the alcohol removed The precipitate is dissolved in 15 ml of water sodium hydroxide added to pH 8 4 trypsin added and the mixture incubated at 37°C for 36 hours with continuous stirring The pH is adjusted to 8 4 by adding dilute NaOH For the first hour the pH must be checked every few minutes with a Beckman glass electrode Preservative is then added and the digestion continued for 36 hours The mixture is acidified (HCl to pH 6 0) and two volumes of 95 per cent ethanol added The precipitate is removed by centrifuging and then extracted by repeated washing with hot acetone until all fat is removed This gives a crude heparin powder which can be subjected to identity tests However unless the tissue has a high heparin content, it is necessary to carry out further purification procedures For quantities of tissue greater than 500 Gm it is necessary to substitute filtration for centrifugation

For identification studies in most tissues it is necessary to process 50 to 75 pounds of tissue When this is done the extractions are carried out in a double jacketed steam kettle (Dopp) Filtration through cotton cloth is used at each stage The heating at various stages of filtration and centrifugation keep the fat fluid so that it will be discarded in the filtrate

Purification depends upon the particular tissue and species being investigated Purification procedures commonly used are (1) treatment with ammonium carbonate (2) charcoal (3) Lloyd s reagent (4) benzidine (5) precipitation as brucine salt and (6) precipitation as the barium salt

Ammonium Carbonate The crude material is dissolved in alkaline

water at an approximate concentration of 6 per cent, 10 per cent ammonium carbonate added heated to 70 C and either centrifuged or filtered through celite on cloth on a Buchner funnel Before further stages the carbonate is removed by slowly adding acetic acid care being taken not to lose solution by frothing This is one of the most useful methods for treating heparin

Charcoal Charcoal is useful for removing coloring matter Charcoal approximately equal to the original weight of crude is added the mixture heated to 70 C and after contact from 30 minutes to five hours is filtered with celite Time of contact must be carefully controlled by trying small samples since heparin can be absorbed by charcoal There is great variation in different samples of charcoal in this regard British Drug House charcoal is satisfactory but each batch must be standardized The charcoal treatment may be repeated adding 1 per cent acetic acid before each treatment

Lloyd s Reagent Originally used by Howell for heparin is one of the most useful materials since it rarely absorbs heparin Best results are obtained if the solution is on the acid side and left in contact with the Lloyd s in the cold overnight Five per cent Lloyd s reagent is added and removed either by centrifugation or filtration through linen–paper pulp–paper This may be repeated several times adding one per cent acetic acid between each treatment

Benzidine This is used to precipitate heparin, but some purification of crude heparin must be achieved before applying benzidine It is best to apply this process to material which is running 20 to 40 per cent heparin The solution is acidified (clear) and five per cent benzidine HCl is added in fractions until no further precipitate is obtained with the benzidine The precipitate is centrifuged supernatant discarded and the precipitate washed with a diluted solution of benzidine The precipitate is hydroly ed by adding water and concentrated ammonia and heating to 70 C During the heating the mixture is checked frequently with litmus The litmus should turn blue and then the spot slowly turn red on standing due to hydrolysis of the benzidine salt The solution must be kept strongly alkaline When hydrolysis is completed it is chilled to 30 C and the benzidine removed by centrifuging and filtering it through linen–pulp–linen Some trace of benzidine remains in the heparin solution and this can be removed by absorption on Lloyd s reagent which oxidizes the benzidine to an insoluble blue compound

Brucine To precipitate the brucine salt of heparin a half volume of 5 per cent brucine phosphate pH 5 5 is added to the heparin solution at pH 5 5 The precipitate is collected and can be washed and dried with absolute alcohol and ether or hydrolysed directly It is hydrolysed in 0 05N NaOH at 70 C for 15 minutes (see Jaques Monkhouse and Stewart) [8]

Barium The barium salt of heparin in concentrated solution is soluble

warm and insoluble cold This can be made use of for precipitating heparin when the heparin concentration reaches 20 to 40 per cent Once purification to the stage of 40 per cent heparin is reached, then crystallization is the method of choice This gives the highest degree of purification in a single step of any of the methods For crystallization the heparin must be treated with ammonium carbonate to remove calcium and barium and to form the ammonium salt The heparin is dissolved one gram per 5 ml of alkaline water, 1 5 ml of 20 per cent ammonium carbonate solution is added the solution heated to 65°C and then centrifuged The precipitate is washed with a 6 per cent solution of ammonium carbonate solution at 65°C, the wash added to the original supernatant and the whole acidified with acetic acid, care being taken at this stage not to lose solution due to rapid evolution of CO_2 The volume is then made up to 12 5 ml and samples are removed for crystallization One to two ml samples are diluted to 5 ml, 1 4 ml of 10 per cent barium acetate solution added and the tube is heated to 65°C The precipitate at this stage is barium sulfate and is removed by centrifuging The clear solution is then warmed to 65°C and 1 2 ml of glacial acetic acid added This is then allowed to cool slowly to room temperature A heavy precipitate comes out starting at the bottom and the center of the tube at about 40 to 45°C Under the microscope it appears crystalline in forms of rosettes and sheaves Depending upon the purity of the sample, the impurities present and the species of heparin, the condition of crystallization may require adjustment The chief significant factors in this regard are concentration of barium acetic and heparin When other trials are required the concentrations of barium and acetic should be varied in steps of about 10 per cent With different species of heparin the final concentration of heparin required varies from 1–6 per cent For preservation of heparin samples for future reference it is advisable to convert them to the neutral sodium salt To do this, the barium is removed with ammonium carbonate as described for preparing for crystallization the carbonate removed by adding acetic acid the solution then carefully adjusted to pH 7 0–7 1 with NaOH and the heparin precipitated by pouring the solution into three volumes of 95 per cent methyl alcohol The resulting precipitate is centrifuged washed twice with ethanol washed twice with ether and dried This form of heparin is quite stable Heparin is hygroscopic and should be stored in tightly sealed containers Dried over calcium chloride at 25°C, it contains 12–15 per cent of water of crystallization In comparing samples of heparin for specific activity allowance must be made for water and base content of each sample Thus water and barium make up approximately 35 per cent by weight of the barium salt

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3 Antithromboplastin Preparation and Assay

R T CARROLL and L M TOCANTINS

By the term *antithromboplastin* is understood an activity present in the blood and extracts of certain tissues directed against the clot accelerating action of tissue or blood thromboplastin The exact point of action of the substance or substances responsible for this activity is not entirely clear As recovered from the tissues by the method to be described, the substance is a lipid in order to exert its action a protein co factor present in the plasma seems necessary What this co-factor is is not known It seems that the lipid inhibitor interferes with the formation of blood thromboplastin as well as antagonizes its action after it is formed

Two forms of this inhibitor have been recognized (a) the antithromboplastic lipoprotein of the blood and perhaps of some tissues and (b) lipid antithromboplastin recovered by extraction of brain blood and certain tissues with fat solvents This section deals chiefly with the latter type

Principle The acetone dried tissue is extracted with absolute methanol the evaporated residue suspended in 0 85 per cent NaCl homogenized by exposure to ultrasonic vibration and assayed in an activated plasma clotting system using a suspension of lipid inhibitor of known potency as a reference standard

Preparation of Tissues for Extraction The organs are obtained fresh without fixatives preferably from a body after accidental death Organs showing gross pathologic changes or from individuals with chronic debilitating diseases are avoided The organs are freed of vessels adherent blood clots or other foreign material and rinsed in 0 85 per cent NaCl In the case of the brain the meninges and vessels must first be removed completely before the saline rinsing If the placenta is used it is perfused with 0 85 per cent NaCl injecting it directly into the placental artery until a fairly clear solution returns Following the rinsing if the organs are not to be

warm and insoluble cold This can be made use of for precipitating heparin when the heparin concentration reaches 20 to 40 per cent Once purification to the stage of 40 per cent heparin is reached, then crystallization is the method of choice This gives the highest degree of purification in a single step of any of the methods For crystallization the heparin must be treated with ammonium carbonate to remove calcium and barium and to form the ammonium salt The heparin is dissolved one gram per 5 ml of alkaline water, 1 5 ml of 20 per cent ammonium carbonate solution is added, the solution heated to 65°C and then centrifuged The precipitate is washed with a 6 per cent solution of ammonium carbonate solution at 65°C, the wash added to the original supernatant and the whole acidified with acetic acid, care being taken at this stage not to lose solution due to rapid evolution of CO_2 The volume is then made up to 12 5 ml and samples are removed for crystallization One to two ml samples are diluted to 5 ml, 1 4 ml of 10 per cent barium acetate solution added and the tube is heated to 65°C The precipitate at this stage is barium sulfate and is removed by centrifuging The clear solution is then warmed to 65°C and 1 2 ml of glacial acetic acid added This is then allowed to cool slowly to room temperature A heavy precipitate comes out, starting at the bottom and the center of the tube at about 40 to 45°C Under the microscope it appears crystalline in forms of rosettes and sheaves Depending upon the purity of the sample the impurities present and the species of heparin the condition of crystallization may require adjustment The chief significant factors in this regard are concentration of barium acetic and heparin When other trials are required the concentrations of barium and acetic should be varied in steps of about 10 per cent With different species of heparin the *final concentration of heparin required varies from 1–6 per cent* *For preservation* of heparin samples for future reference it is advisable to convert them to the neutral sodium salt To do this, the barium is removed with ammonium carbonate as described for preparing for crystallization the carbonate removed by adding acetic acid the solution then carefully adjusted to pH 7 0–7 1 with NaOH and the heparin precipitated by pouring the solution into three volumes of 95 per cent methyl alcohol The resulting precipitate is centrifuged washed twice with ethanol washed twice with ether and dried This form of heparin is quite stable Heparin is hygroscopic and should be stored in tightly sealed containers Dried over calcium chloride at 25°C it contains 12–15 per cent of water of crystallization In comparing samples of heparin for specific activity allowance must be made for water and base content of each sample Thus water and barium make up approximately 35 per cent by weight of the barium salt

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warm and insoluble cold This can be made use of for precipitating heparin when the heparin concentration reaches 20 to 40 per cent Once purification to the stage of 40 per cent heparin is reached, then crystallization is the method of choice This gives the highest degree of purification in a single step of any of the methods For crystallization the heparin must be treated with ammonium carbonate to remove calcium and barium and to form the ammonium salt The heparin is dissolved one gram per 5 ml of alkaline water, 1 5 ml of 20 per cent ammonium carbonate solution is added, the solution heated to 65 C and then centrifuged The precipitate is washed with a 6 per cent solution of ammonium carbonate solution at 65°C the wash added to the original supernatant and the whole acidified with acetic acid, care being taken at this stage not to lose solution due to rapid evolution of CO_2 The volume is then made up to 12 5 ml and samples are removed for crystallization One to two ml samples are diluted to 5 ml, 1 4 ml of 10 per cent barium acetate solution added and the tube is heated to 65°C The precipitate at this stage is barium sulfate and is removed by centrifuging The clear solution is then warmed to 65°C and 1 2 ml of glacial acetic acid added This is then allowed to cool slowly to room temperature A heavy precipitate comes out, starting at the bottom and the center of the tube at about 40 to 45°C Under the microscope it appears crystalline in forms of rosettes and sheaves Depending upon the purity of the sample the impurities present and the species of heparin the condition of crystallization may require adjustment The chief significant factors in this regard are concentration of barium acetic and heparin When other trials are required the concentrations of barium and acetic should be varied in steps of about 10 per cent With different species of heparin the final concentration of heparin required varies from 1–6 per cent For preservation of heparin samples for future reference it is advisable to convert them to the neutral sodium salt To do this, the barium is removed with ammonium carbonate as described for preparing for crystallization the carbonate removed by adding acetic acid, the solution then carefully adjusted to pH 7 0–7 1 with NaOH, and the heparin precipitated by pouring the solution into three volumes of 95 per cent methyl alcohol The resulting precipitate is centrifuged washed twice with ethanol washed twice with ether and dried This form of heparin is quite stable Heparin is hygroscopic and should be stored in tightly sealed containers Dried over calcium chloride at 25 C, it contains 12–15 per cent of water of crystallization In comparing samples of heparin for specific activity allowance must be made for water and base content of each sample Thus water and barium make up approximately 35 per cent by weight of the barium salt

cipitation, the residue is dissolved in absolute ethyl ether and the ether solution transferred to a weighed beaker The ether is removed *in vacuo* This material is whiter and flakier than the cruder product

Assay The fractions as they are obtained from the tissues are weighed accurately and the residue placed in a small mortar To this is added the correct volume of 0 85 per cent NaCl to make a 1 per cent solution The mixture is rotated with a small pestle until an even suspension (or a com plete solution) is accomplished It is then put through a small hand oper ated homogenizer seven times The inhibitor potency appears to be directly related to particle size and dispersion every time it is allowed to go through the homogenizer its clot delaying activity is enhanced until a maximum effect has been achieved (table 1) As can be seen from table 1, the potency of the inhibitor is increased until a maximum is reached at seven homogen izations when it levels off The potency may be further enhanced by ex posing the material for a given period at a definite frequency to the Ray theon supersonic vibrator (9 kilocycles 50 watts type K 223 serial # 317) The minimum volume to be exposed should not be less than 5 ml Below this volume the results are not reproducible If the correct volume of a given solution is exposed in the vibrator at a definite frequency for a uni form period of time duplicate results can be obtained Different vibrators do not give the same results As in the case of the hand homogenizer, the longer the period of exposure the greater the enhancement of potency until a maximum effect has been achieved (table 1)

For uniformity in handling and comparing with standards 5 ml of a 1 per cent suspension exposed to the ultrasonic vibrator (9 kc) for a period of twenty minutes is always used Following homogenization the material is maintained at 5 C until ready for testing If the material is to be kept over a longer period than 4-6 hours for additional testing it should be re homogenized in the ultrasonic vibrator The standard should of course be treated in the same manner Just prior to testing the suspen sions are brought to room temperature and using 0 1 N NaOH adjusted to pH 7 2 to 7 4 with a Beckman pH meter Prior to adjustment of pH the suspension is usually slightly acid (6 5-6 8)

Preparation of reagents for the assay (1) *Tissue thromboplastin* 300 mg of acetone dried human brain powder prepared as previously described are extracted for thirty minutes with 5 ml of 0 85 per cent sodium chloride solution in a 50 ml round bottom glass tube placed in a water bath at 48 C The tube is rotated for a few seconds at 5 minute intervals The tube is then centrifuged at 1500 rpm for two minutes and the supernatant fluid removed by decantation centrifuged again in a similar fashion and the somewhat cloudy supernatant decanted off and used A large batch of the thromboplastin is prepared at one time amounts from 2-3 ml placed in

dried immediately, they are placed in a deep freeze at −10°C and maintained in the frozen state until ready for the next step, i e , the drying process

Drying The fresh or thawed out organ is removed from the refrigerator in 30–50 gram portions placed in a mortar and cut into small pieces To these is then added approximately 150 ml C P or technical grade acetone and the organ is macerated with a pestle using a constant rotatory, cru h ing motion When it is felt that the maximum dehydrating effect has been achieved with the acetone sample in use, this is discarded by decantation and fresh acetone is added The amount of acetone required depends on the initial ' wetness of the tissue as well as its consistency The above procedure is repeated until a fine grayish white powder is obtained This is then spread on a large, dry flat piece of glass and the residual acetone removed by evaporation This process is hastened somewhat by frequent changing of the exposed surface by means of a spatula constantly spreading and tamping Excessive exposure to air during the drying process should be avoided The dried powder is then passed through a #20 mesh wire screen and is ready for further processing

Extraction To 20 grams of the acetone dried powder previously described is added 800 ml C P absolute methanol and the mixture allowed to remain in a wide vessel at 5°C for five days, with occasional shaking The supernatant solution is filtered through two thicknesses of Whatman #2 filter paper and the filtrate distilled *in vacuo* in a flask immersed in an H_2O bath maintained at 45°C The brownish residue remaining in the distilling flask is removed with absolute ethyl ether the ether solution then being placed in a 50 ml round bottom centrifuge tube at 5 C where it may remain from 6–12 hours during which a white precipitate settles out The mixture is centrifuged at 1500 rpm for 3 minutes the supernatant is decanted off and the precipitate washed once with cold absolute ether The combined ether extracts are evaporated *in vacuo* leaving a creamy yellow, waxy powder This is the *crude antithromboplastin*

Purification 200 mg of this waxy powder is placed in a 40 ml round bottom centrifuge tube and dissolved in 4–5 ml absolute ethyl ether To this solution is then added sufficient cold absolute ethanol to achieve maximum precipitation usually 10–15 ml The ethanol is added slowly with constant swirling of the tube The mixture is placed at 5 C for thirty minutes and then centrifuged at 1500 rpm for two minutes The supernatant is removed by decantation and the residue dissolved in 4–5 ml absolute ethyl ether Again cold absolute ethanol is added until maximum precipitation is achieved The above is repeated until a minimum of five precipitations have been accomplished The greater the number of precipitations the greater the potency of the inhibitor Following the final pre

cipitation, the residue is dissolved in absolute ethyl ether and the ether solution transferred to a weighed beaker The ether is removed *in vacuo* This material is whiter and flakier than the cruder product

Assay The fractions as they are obtained from the tissues are weighed accurately and the residue placed in a small mortar To this is added the correct volume of 0 85 per cent NaCl to make a 1 per cent solution The mixture is rotated with a small pestle until an even suspension (or a complete solution) is accomplished It is then put through a small hand operated homogenizer seven times The inhibitor potency appears to be directly related to particle size and dispersion every time it is allowed to go through the homogenizer its clot delaying activity is enhanced until a maximum effect has been achieved (table 1) As can be seen from table 1 the potency of the inhibitor is increased until a maximum is reached at seven homogenizations when it levels off The potency may be further enhanced by exposing the material for a given period at a definite frequency to the Raytheon supersonic vibrator (9 kilocycles 50 watts type K 223, serial # 317) The minimum volume to be exposed should not be less than 5 ml Below this volume the results are not reproducible If the correct volume of a given solution is exposed in the vibrator at a definite frequency for a uniform period of time duplicate results can be obtained Different vibrators do not give the same results As in the case of the hand homogenizer the longer the period of exposure the greater the enhancement of potency until a maximum effect has been achieved (table 1)

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TABLE 1 *Effect of Hand Operated Homogenization and Ultrasonic Vibration on Activity of Lipid Antithromboplastin*

No of Times 1% suspension of inhibitor passed through hand homogenizer	No of Minutes exposed to ultrasonic vibration	Activated Clot Time (seconds)
0		55
2		68
4		74
5		88
7		92
10		92
	0	58
	1	122
	2	161
	5	191
	10	218
	15	241
	20	246
Control†		15

* 0 1 ml 1 per cent inhibitor suspension 0 1 ml thrombopl 0 1 ml citrated plasma 0 1 ml 0 02 M $CaCl_2$

† 0 85 per cent NaCl replacing inhibitor suspension

cork stoppered glass tubes quick frozen with dry ice and kept in the deep freeze at −10 C until ready for use It is then thawed rapidly by repeated immersion for a few seconds at a time under hot running water Once the thromboplastin has been thawed, it retains its potency for 6–8 hours if placed in the refrigerator at 5°C when not in use When needed, it is brought to room temperature diluted if desired then added to the testing mixture A thromboplastin solution cannot be repeatedly thawed without losing some of its potency If prepared and kept as described the thromboplastin solutions maintain a constant potency for months As much as possible reagents prepared from the same animal species should be used For example if human lipid antithromboplastin is being tested, human thromboplastin and human plasma should be used in the testing mixture It is also preferable that a dilute thromboplastin be used if the unknown fraction possesses only slight anticoagulant activity since a concentrated thromboplastin may well overwhelm a weak inhibitor Generally speaking if the original thromboplastin solution is diluted 1–20 with 0 85 per cent NaCl before adding it to the testing mixture an adequate clot accelerating effect is obtained

(2) *Plasma* Blood is collected from a normal donor through a #18

needle into a siliconized syringe containing one hundredth volume of 38 per cent sodium citrate If the initial venepuncture is not a clean ' one the blood collected is considered to be worthless and a fresh specimen is collected preferably from a different vein To insure a good venepuncture the flow of blood into the syringe should be at the rate of at least 1 cc / second Following collection the syringe is tilted back and forth slightly to insure proper mixing with the citrate and then the blood is carefully placed in silicone-coated tubes removing the needle before allowing the blood to run down the side of the tube The tube is then placed in a refrigerated centrifuge (temperature maintained at 5 C) and centrifuged at 4500 rpm for one hour The upper ¾ of the plasma is then carefully removed by means of a siliconized dropper pipet into a siliconized tube care being taken not to disturb the lower layers of plasma The tube is stoppered and refrigerated at 5 C until ready for use on the same day It is then brought to room temperature and added to the clotting mixture using a 1 0 ml siliconized pipet graduated in 0 01 ml

Testing Silicone-coated glassware is used in preparing and measuring the plasma The clotting mixture is made up of the following ingredients, *always* added in this order to silicone coated tubes at 38 C

0 1 ml unknown solution (or 0 85 per cent NaCl)
0 1 ml human thromboplastin
0 1 ml normal citrated human plasma
0 1 ml 0 02 M $CaCl_2$

Calculation The activity of the unknown is expressed in terms of units per mg by comparing it with the activity of a lot of lipid antithrom boplastin kept as a reference standard A partially purified lipid antithrom boplastin obtained from human brain has been used as the standard In the dry state at −10 C the lipid has maintained its original anticoagulant activity for at least two years when compared in an activated clotting system with another anticoagulant (Toronto heparin 100 units per mg) One gram of a purified lot of the lipid inhibitor was selected as a standard and granted arbitrarily 20 units of antithromboplastin activity per mg When a lipid extract is to be assayed for antithromboplastin activity 50 mg of the standard is mixed with 5 ml of 0 85 per cent NaCl and after hand homogenization exposed to the water-cooled ultrasonic vibrator for 20 minutes The unknown sample is prepared in the same manner A con centration/activity curve for the standard antithromboplastin is worked out each time utilizing as a clotting substrate the same plasma and throm boplastin employed in assaying the antithromboplastin activity of the unknown extract In figure 1 the line is plotted which expresses the rela tionship between activity (clotting time) and amount of antithrombo- plastin of a partially purified material from dried human brain

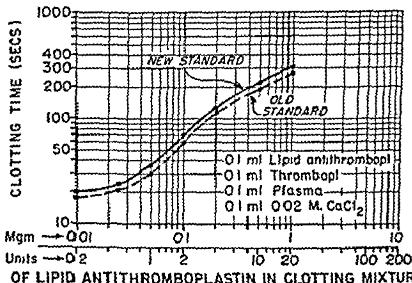


FIG 1—Titration curve of a new standard lipid inhibitor on the basis of the curve for the old standard The curve of activity of the new standard between 40 and 200 seconds is used for interpolating values obtained in testing the unknown samples Clotting times are converted into units of activity by reading the values in the abscissa corresponding to each time

As can be seen by a study of the curve the concentration/activity ratio plotted on a logarithmic scale is not linear throughout For purposes of interpolating values obtained from unknown samples being assayed for activity the straight portion of the curve between 40 and 200 seconds on the chart is used If the unknown sample has an initial clotting time above (or below) these limits it is diluted (or stronger solutions are used) so that the values fall into the useful section of the curve

Example for calculating the units of antithromboplastin activity of an unknown sample The reagents (thromboplastin plasma lipid inhibitor and 0 02 M CaCl solution) are prepared as described Silicone-coated glassware is used throughout Measurement of the clotting time is done in duplicate at 38 C

Tube No 1 0 1 ml 0 85 per cent NaCl 0 1 ml thromboplastin 0 1 ml citrated plasma 0 1 ml 0 02 M $CaCl_2$

Tube No 2 0 1 ml 1 *per cent suspension of the new standard lipid* inhibitor 0 1 ml thromboplastin 0 1 ml citrated plasma 0 1 ml 0 02 M $CaCl_2$

Tubes No 3 4 5 6, 7 and 8 Same as tube No 2 except that the standard inhibitor solution is diluted with 0 85 per cent NaCl to obtain respectively the following concentrations 0 5 0 2 0 1 0 05 0 02 0 01 per cent The clotting times of the mixtures in tubes 2 to 8 are plotted on the chart (fig 1)

Tube No 9 0 1 ml 1 per cent suspension of *unknown sample* 0 1 ml thromboplastin 0 1 ml citrated plasma, 0 1 ml 0 02 M $CaCl_2$

Let us say that the clotting time of the mixture in tube No 9 is 52 seconds This is the activity of 1 mg of the unknown sample To convert this value to units of the standard the point on the abscissa is located which corresponds to 52 seconds on the new standard curve (Fig 1) This is equivalent to 1 6 units of antithromboplastin activity or the activity found in 0 08 mg of the standard inhibitor It may be said then that the unknown sample has 1 6 units of antithromboplastin activity (A U) per mg

In order to assay the *total* units of antithromboplastin activity in a given sample of blood, plasma or tissue the yield of the extract in mgms is multiplied by *the units of activity per mg* of *the extract*

Before the supply of standard inhibitor is exhausted a new lot should be prepared and standardized in terms of the old one A relatively large amount of lipid inhibitor should be prepared in order to eliminate the necessity for frequent restandardizations To obtain a large yield 30 grams of dried brain powder is used as a start and to it are added 1200 ml of absolute methanol Following the filtration distillation removal with absolute ethyl ether and the separation of the ether insoluble material in the refrigerator the final yield should be 2 0 to 2 5 grams A 1 per cent suspension is prepared in 0 85 per cent NaCl homogenized and an activity curve is worked out as previously described At the same time using the same thromboplastin and plasma as basic reagents an activity curve is worked out for the old standard lipid inhibitor The two curves (fig 1) are compared and a new unitage is granted to the new lot in terms of the units of the old standard

Precautions and Sources of Error (a) Use of technical grade methanol instead of *C P Absolute Methanol* in the extracting procedure (b) Extraction at room temperature if extraction is allowed to proceed at temperatures higher than 10 C coagulant materials are extracted and the anticoagulant potency of the assayed materials is lower (c) Imperfect homogenization of the suspension If the lipid suspension is not rendered almost water clear by this process the ultrasonic homogenizer is probably defective (d) Allowing material to stand too long (over 2 hours) after homogenization before it is tested (e) Use of heterologous thromboplastins or plasma substrates (f) Use of unstable hypercoagulable plasmas as substrates

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APPENDIX

1 Synonyms for Components Influencing Blood Coagulation*

Group 1

Thromboplastinogenase
Thromboplastic cell component (TCC)

Group 2

Prothrombokinase
Plasmakinin
Antihemophilic globulin
Thromboplastinogen
Thrombocytolysin
Thrombokatalysin
Thromboplastic plasma component (TPC)
Factor VIII
Antihemophilic Factor

Group 3

Plasma thromboplastin component (PTC)
Christmas Factor
Factor IX
Platelet cofactor

Group 4

Thrombokinase
Cytozyme
Thromboplastic protein
Thrombokinin
Plasma thromboplastin

Group 5

Thrombogene
Component A of prothrombin

* When a precursor is postulated for a factor the precursor is listed on the left and the corresponding active factor on the right

Factor V
Factor VI
Accelerator factor
Labile factor
Cofactor of thromboplastin

Plasma Ac globulin	Serum Ac globulin
Proprothrombinase	Prothrombinase
Prothrombinogenase	Thrombinogenase
Plasma prothrombin conversion factor (PPCF)	Serum accelerator
Proaccelerin	Accelerin

Group 6

Cofactor V
Prothrombin accelerator
Prothrombin conversion factor
Prothrombin converting factor
Co thromboplastin

Plasma precursor	Serum prothrombin conversion accelerator (Spca)
Proconvertin	Convertin

Factor VII
Kappa factor (in chicken)

Group 7

Prothrombin
Prothrombase
Thrombozyme

Group 8

Thrombin
Thrombase

Group 9

Antithromboplastin
Thromboplastin inhibitor
Antithrombokinase

Group 10

Antithrombin
Thrombin inhibitor

Group 11

Plasminogen	Plasmin
Profibrinolysin	Fibrinolysin
Tryptogen	Tryptase (serum tryptase)
Prolysin	Lysin
Lytic factor	

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Factor V — Factor VI

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Thrombase

Group 9

Antithromboplastin
Thromboplastin inhibitor
Antithrombokinase

Group 10

Antithrombin
Thrombin *inhibitor*

Group II

Plasminogen	Plasmin
Profibrinolysin	Fibrinolysin
Tryptogen	Tryptase (serum tryptase)
Prolysin	Lysin
	Lytic factor

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